



PHD

**Formulation and Delivery of Topically  
Applied Drugs for the Treatment of Atopic  
Eczema and Other Related Diseases**

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# **FORMULATION AND DELIVERY OF TOPICALLY APPLIED DRUGS FOR THE TREATMENT OF ATOPIC ECZEMA AND OTHER RELATED DISEASES**

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath  
Department of Pharmacy and Pharmacology  
December 2010

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## Abstract

Atopic eczema is an incurable disorder of the skin. Sufferers are afflicted with hypersensitivity to environmental agents such as soaps (detergents), animal dander, pollen, specific foods and sometimes even water. Genetic mutations in atopic eczema compromise the development of the stratum corneum resulting in xerotic skin that is prone to cracking and increased permeability which leads to irritation due to the influx of exogenous material through the skin. The causes of atopic eczema are due to a combination of genetic and environmental factors and it is, therefore, a difficult disease to manage. Emollients and topical corticosteroids are the mainstay treatments for eczema. However, they do not treat the underlying cause of the flare-ups frequently seen in the condition; the damaged skin barrier. Defects in the skin barrier arise from premature desquamation of the stratum corneum. The main contributors to barrier breakdown are the up-regulation of skin proteases that are located in the skin. Since zinc is a known protease inhibitor, it would thus follow that a topical treatment for skin barrier repair should be developed. Therefore, the main objectives of this thesis are to successfully incorporate zinc into a formulation to develop a novel class of treatment for eczema and to assess the delivery of the element into the skin.

In this thesis, methods to assess and characterise changes to skin barrier function and to extract and quantify zinc in the stratum corneum have been established. The development of two novel topically applied formulations containing zinc lactate as the active ingredient (1% w/w zinc lactate cream and a 2% w/v zinc lactate formulation) has been achieved and the uptake of zinc from the preparations *in vitro* determined. Further, the *in vitro* percutaneous penetration of zinc from three commercially available preparations has been investigated and compared to that recovered from the stratum corneum after passive diffusion with the novel zinc formulations. Additionally, *in vivo* uptake of zinc into human stratum corneum from Sudocrem<sup>®</sup> is reported. Scanning electron microscopy has revealed the distribution of zinc on the surface of skin treated with various formulations and has also allowed the efficiency of two cleaning procedures to be ascertained.

The delivery of zinc from the novel topical formulation; 1% w/w zinc lactate cream, was more efficient than that the three commercial formulations and shows promise as a new approach to treat atopic eczema.





## **Chapter One: Introduction**



## **Chapter One**

### **Introduction**

Atopic eczema (AE) is the most common skin disorder in the United Kingdom and accounts for approximately 30% of dermatological consultations. It is estimated that 9% of the UK population is affected by AE at any one time and at least 40% of people will have at least one episode during their lifetime.(1, 2) The average age of onset of AE is 3-7 months, with approximately 25% of infants under the age of five, and 2-10% of adults affected.(1-5) The prevalence of AE has a higher frequency in urban rather than rural areas and also in smaller families with higher socioeconomic backgrounds which suggests that the development of AE may be due to the lack of exposure to infectious agents or antigenic triggers and increased contact with antigenic pollutants.(6, 7)

AE is a highly distressing, chronic, recurring inflammatory disorder of the skin. Affected individuals are hyperreactive to environmental triggers that are normally innocuous to healthy individuals with no history of hypersensitivity.(8) In infants, the effects are initially presented on the face and eventually advance to the rest of the body (Fig. 1) .(2, 3) Areas of skin affected typically include the neck, scalp, trunk and flexures of the arms and behind the knees.(2-4) Sufferers present with xerotic, erythematous, oozing lesions which are intensely pruritic and simultaneously excruciating. The pruritic characteristics of eczema induce scratching which can result in the formation of lesions and, in some cases, bleeding. Lichenification and excoriation are also commonly observed features of eczema.(9)



Fig. 1 An example of facial eczema in an infant (10)

AE significantly affects the quality of life for affected individuals and their families. Children (and adults) suffer from sleep-disturbance and, in some cases, the disease may have a severe impact on their physical, social and psychological development since the areas affected, the face, neck and flexural positions of the arms and legs, are visible and often associated with disfigurement.(11, 12) A study involving paediatric nurses showed that even medical professionals showed signs of avoidance to children affected with eczema. The psychological impact of this on children may be long-lasting and carry on into adolescence or adulthood.(12) Research has also revealed that preschool children affected with eczema tend to have higher rates of behavioural problems and showed more fearfulness, emotional attachment and dependency on their parents.(13) In the case of children of school-age, those affected with moderate to severe eczema exhibited twice the rate of psychological disturbance than their non-atopic peers.(14) These disturbances can be partly attributed to bullying, unwanted comments and frequent feelings of embarrassment in social settings which may eventually lead to social isolation and depression.(15) Not only is the child affected by AE, but the parents also suffer physical and mental exhaustion by caring for the restless child throughout the night and are plagued by feelings of hopelessness, guilt, frustration and depression.(15, 16) Though AE is not perceived as a serious medical condition, it can have a significant impact on quality of life. Limitations on the child's daily activities, for example, going on holidays, choice of clothing, owning of pets, all contribute to decrease the quality of life experienced by AE sufferers.(15) A study comparing the quality of life in children affected with dermatological disorders and others with chronic childhood diseases found that AE and psoriasis had the greatest impact on children with skin disorders. Of all chronic childhood diseases, only cerebral palsy scored higher than AE.(17)

AE is affiliated with a number of other allergies such as allergic rhinitis, asthma, food allergies and hayfever. This is known as the 'Atopic March' (AM).(6) There is controversy surrounding whether the AM really does exist and if it can be prevented.(18) However, there is compelling evidence that suggests otherwise. Some researchers believe that the AM is '...the natural history of atopic manifestations, characterised by a typical sequence of progression of clinical signs of atopic disease...' and consider AE as the basis of the AM which subsequently leads to the development of asthma and other allergic diseases.(6) It has been reported that 30-60% of paediatric patients will progress on to develop other allergies alongside eczema.(19) Other research suggests that the atopic diseases appear to

‘take turns’ where one becomes more dominant whilst the others subside.(6) Longitudinal studies discovered that as AE improved, 43% and 45% of sufferers proceeded to develop asthma or allergic rhinitis, respectively, within 8 years and that the patients, who did not develop either of these allergies, were only affected with mild AE.(20) This suggests that the risk factor for the development of other allergies, especially asthma, depends on the severity of AE.(6) The study found that 70% of patients affected by severe AE went on to suffer from asthma compared to 30% of those only mildly affected (compared to the general (non-atopic) population of only 8%). A 7-year study involving 1314 children revealed that 69% had developed AE by the age of three months and were sensitised against aeroallergens by the age of five. Those who had higher chances of developing the disease, (i.e., already had 2 family members suffering from atopy) had a 77% risk of sensitisation.(21, 22) Furthermore, a study conducted by Novembre *et al.* involving 77 children affected with intrinsic AE (AE<sub>i</sub>) (diagnosed according to Hanifin and Rajka) concluded that, at 2 years of age, 22% had asthma and 64% tested positive for at least one allergy via skin prick tests (SPT) to food and inhalant allergens. However, at age 11, only 46% still had AE but surprisingly 43% now had asthma and those testing positive to STP had risen to 84%. Interestingly, none of the ‘nonatopic’ children (15% of the total number), that is, those who tested negative for SPT at ages 2 and 11, had developed asthma by the time of the second evaluation.(23) Such evidence of AE being linked to respiratory and food-allergies all share a common pathogenesis and genetic basis and it has been proven in seven linkage studies that these diseases all have 9 different chromosomal regions associated to asthma.(24)

AE is classified into two main categories; the *intrinsic* non-allergic form (AE<sub>i</sub>), and the *extrinsic* IgE-mediated allergic form (AE<sub>e</sub>).(25, 26) The aetiology of both forms is thought to be a multifactorial combination of genetic mutations and a hypersensitivity to environmental stimuli.(8) Scientific evidence suggests that the genetic mutations are responsible for (i) producing a defective skin barrier, and (ii) hyperreactivity to certain external stimuli that are innocuous to healthy non-atopic individuals.(8) According to literature sources, AE<sub>e</sub> is the most prevalent form of the two with approximately 70-85% of AE patients affected. It is associated with high levels of total and allergen-specific IgE in the blood or skin and patients are frequently also affected by other allergic conditions such as allergic bronchial asthma or allergic rhinoconjunctivitis.(27-29) Park *et al* found that all infants with AE<sub>e</sub> had food allergies and only a proportion of these were sensitive to

aeroallergens. Infants who were affected by both forms of allergies had higher total IgE levels than those with only food allergies. This suggests that allergy exposure is proportional to IgE levels.(27) Furthermore, it was discovered that patients with both allergies scored higher on the SCORAD index (severity scoring of atopic dermatitis) as a result of allergen exposure and thus had more severe clinical presentations of the disease (compared to patients affected with AE<sub>i</sub>). (27) With AE<sub>i</sub>, patients test negative to SPT for common food and inhalant allergens, have normal levels of total serum IgE, no detectable specific IgE to food and aeroallergens, and do not suffer from other atopic diseases such as those mentioned previously.(27, 29, 30) Nonetheless, AE<sub>i</sub> patients still possess the typical phenotype of AD and manifest identical skin lesions to AE<sub>e</sub> patients.(27, 28) Between 10-45% of AE patients suffer from this type of the disease.(27) According to Park *et al*, infants diagnosed with AE<sub>i</sub> had lower eosinophil counts and eosinophil cationic protein levels (as with adult patients). They found the opposite to be true with AE<sub>e</sub>. Their results indicate that these parameters are well correlated with the clinical severity experienced by patients and that pathogenesis of AE is affected by the role that eosinophils play in the inflammation of the disease state.(27)

It is evident that the pathology of AE is very complex and is due to a combination of genetic and environmental triggers. Research has shown that AE is not attributed to a single gene but many genes which have been identified and linked to other allergic disorders.(19) The most prominent gene associated with AE is the profilaggrin gene, FLG, that codes for filaggrin.(31) Loss-of-function mutations in FLG have been shown to be a major genetic predisposing factor for AE. These mutations reduce the amounts of profilaggrin produced by the keratohyalin granules in the epidermis.(32) This results in decreased levels of filaggrin (profilaggrin is dephosphorylated and proteolysed to form filaggrin) which,<sup>23</sup> in turn, causes a decrease in the levels of natural moisturising factor (NMF) in the stratum corneum (SC) (NMF is the final product of filaggrin) (Fig. 2).(33) Deficiencies in filaggrin lead to a defective skin barrier since the lack of water-absorbing NMF results in shrinkage of corneocytes causing fissures to appear between them, resulting in increased transepidermal water loss and the influx of pathogens, allergens and xenobiotics into the body leading to flare-ups of AE.(34)

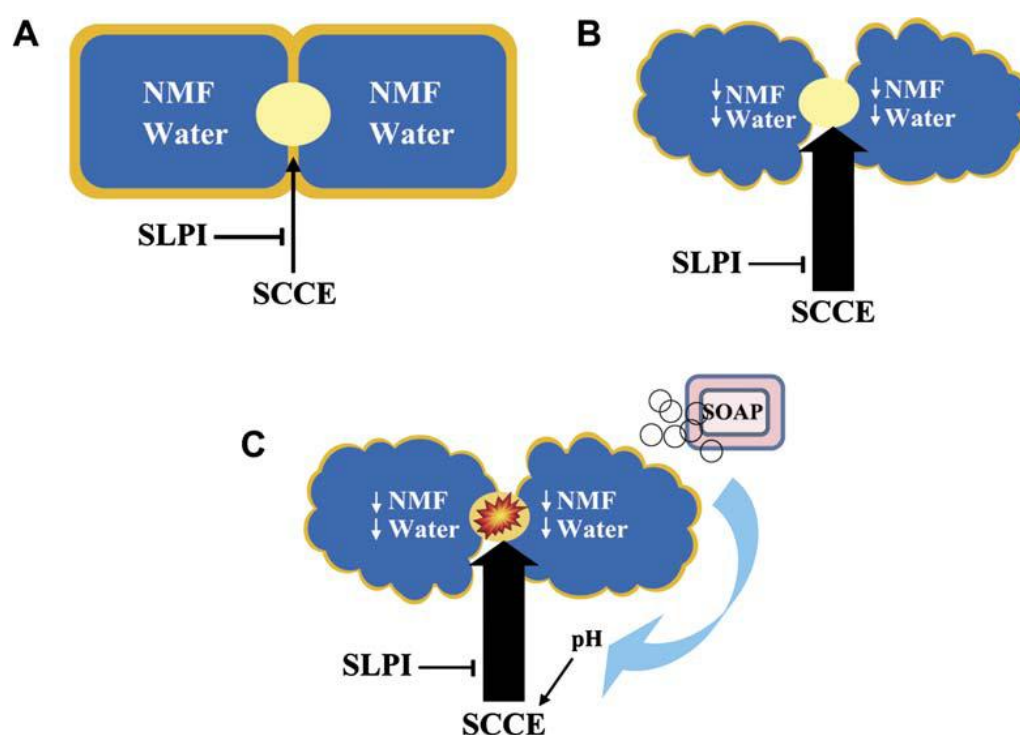


Fig. 2 (A) Normal healthy skin where proteases are in balance with protease inhibitors. Normal production of NMF keeps the SC hydrated resulting in tight-junctions between corneocytes. (B) Genetic predisposition to atopic eczema increases desquamation and decreases the production of NMF. As a result, the stratum corneum is thinner, xerotic and prone to the formation of fissures. (C) Environmental proteases such as soaps and detergents further increase the rate of desquamation and contribute the breakdown of the skin barrier.(35)

Environmental contributions to the manifestation of eczematous lesions via the already damaged skin barrier include increase in the frequency of washing, use of detergents, house-dust mites, pollen, fungal allergens, and the list goes on (Fig. 3). Furthermore, climate change and stress (life-events such as parental divorce or death in the family) also provoke flare-ups.(36, 37)



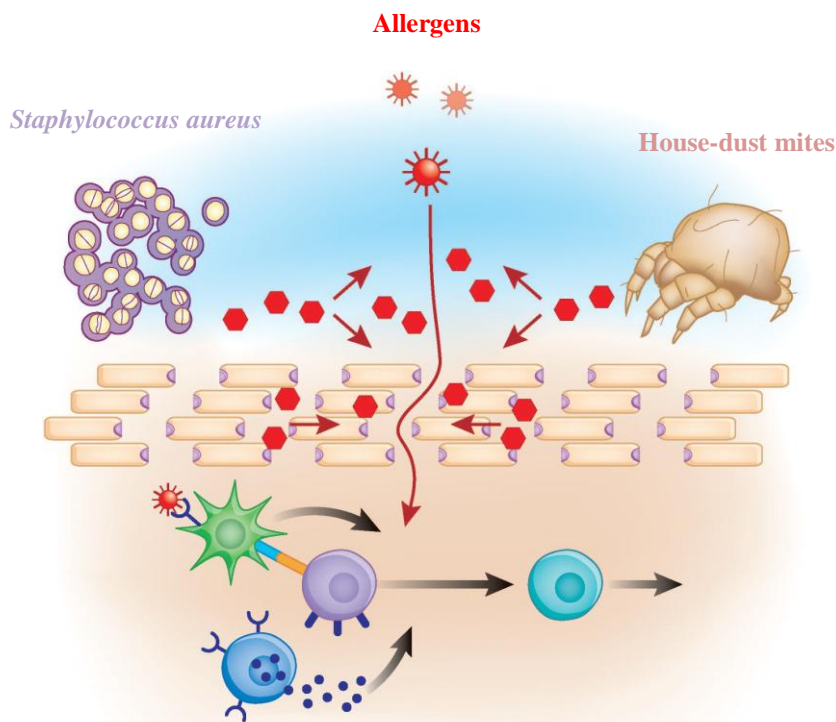


Fig. 3 The defective skin barrier in atopic eczema facilitates the influx of irritants and allergens. Endogenous proteases residing within the epidermis and external proteases contribute the breakdown of the skin barrier. Hyperactive immune responses result in inflammation at the affected sites and together with the proteases lead to the formation of eczematous lesions.(35)

However, the use of detergents probably has the greatest effect on AE suffers when the damaged skin barrier is subjected to washing with hard water and detergents which strip away the lipids in the skin that keep the SC hydrated and flexible (Fig. 4).(38) Furthermore, surfactants contained in the detergents further irritate the skin causing inflammation which results in itching and scratching which then produces lesions. These lesions, due to the protracted healing process, are exposed to these causal factors repeatedly thus rendering skin repair a challenge.

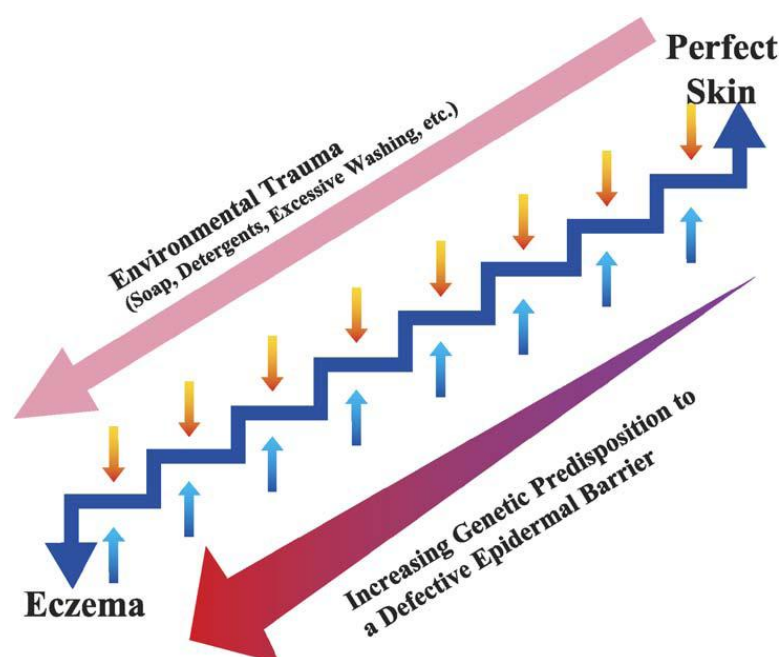


Fig. 4 Detergents and other environmental factors combined with a genetic predisposition to the development of eczema causes the breakdown of the skin barrier resulting in flare-ups of the disease.(8)

Though an individual may have the defective genes that predispose them to AE, physical signs of the disease may not be observed. This is due to the gene-environment dosage interaction where, for example, if an individual has mutations in 5 genes that makes them more inclined to exhibit the disease, it may take fewer environmental factors to trigger it and vice versa - if the individual only has 2 mutations in those genes, then multiple exposures to environmental stimulants will induce the disease state.(8) Absence of manifestations of symptoms may simply mean that the environmental dosage threshold has not been breached.

The skin is a complex structure consisting of four compartments; the hypodermis, dermis, viable epidermis and the SC (Fig. 5).(33)

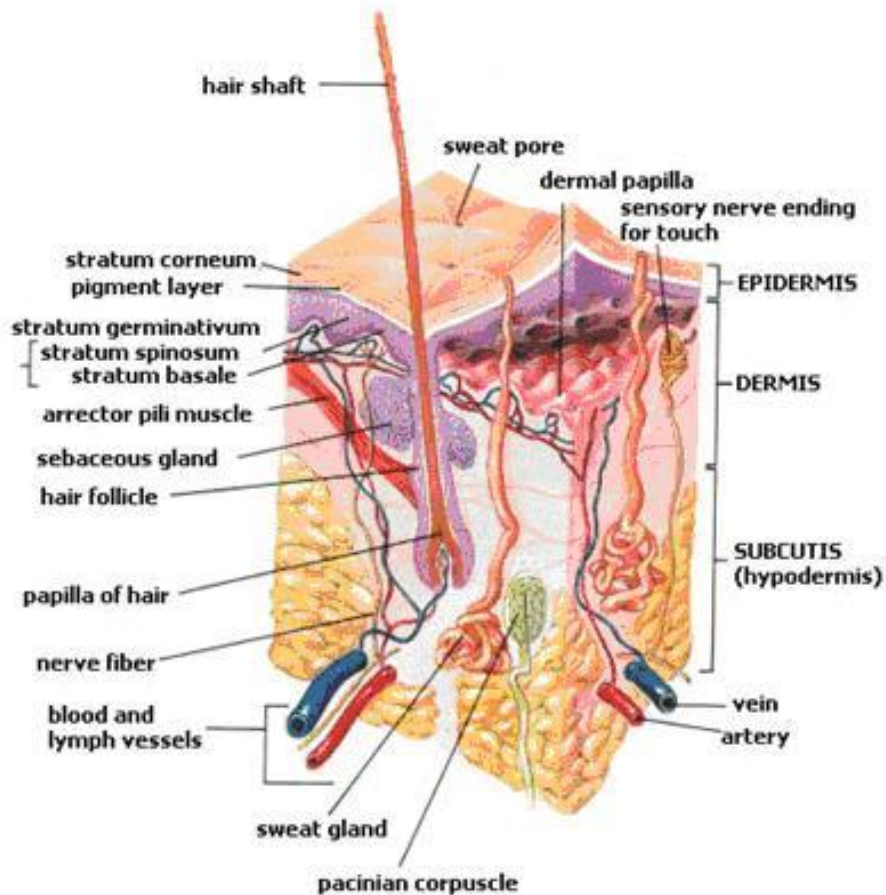


Fig. 5 Structure of the skin (39)

With respect to the body's first line of defence against external assault by foreign substances, the SC is the most relevant. Any breach of this layer would leave the body vulnerable to attack and infection.(8, 40) It forms the barrier to penetration of substances into, and the exit of water out of, the body. It is also the thinnest and smallest of all skin compartments.

The SC consists of corneocytes, corneodesmosomes and the lipid lamellae. With the corneocytes being analogous to bricks, the corneodesmosomes as iron rods, and the lipid lamellae as cement, the SC can be thought of as a brick wall being locked together by the corneodesmosomes and bonded by the lipid lamellae.(41) The corneodesmosomes provide the SC with tensile strength whilst the lipid lamellae help prevent water loss.(41) The homeostasis of the SC is a finely controlled process by which the SC thickness is regulated by proteases and protease inhibitors. Proteases breakdown extracellular corneodesmosomal adhesion proteins that hold corneocytes together to allow the shedding of cells from the SC

surface. In particular, the stratum corneum chymotryptic enzyme (SSCE) and the stratum corneum tryptic enzyme (SCTE) are the two most prominent proteases.(42, 43) SCTE is able to activate SSCE and itself in order to bring about desquamation. The activity of SCCE and SCTE is regulated by protease inhibitors such as the leukoprotease inhibitor.(42, 43) Over-expression of SCCE leads to the premature desquamation of the SC resulting in decreased thickness as basal cell proliferation is not able to replace the lost cells quickly enough and, as a consequence, the skin is more vulnerable to the penetration of irritants and allergens and also to increased water loss. When the barrier is compromised as such, secondary proteases produced by cells within the inflammatory infiltrate are produced which further contribute to desquamation.(8) The level of these proteases is proportional to the severity of the eczematous lesion.(44) In addition, exogenous proteases from house-dust mites and detergents also augment desquamation which, in AE sufferers, is extremely detrimental to the already weakened skin barrier (Fig. 6).

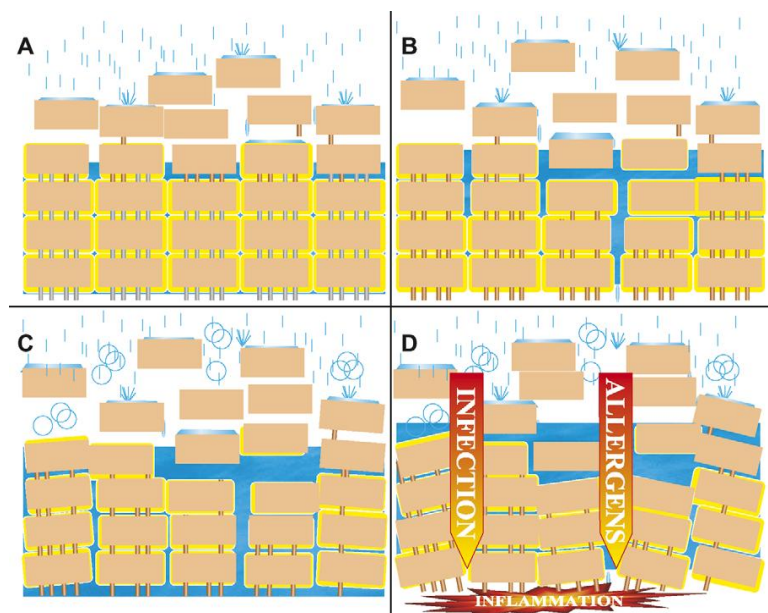


Fig. 6 The stratum corneum is analogous to a brick wall. (A) In a healthy skin barrier the iron rods (corneodesmosomes) breakdown as part of the normal desquamation process. (B) Predisposition to eczema causes the ‘rods to rust’ leading to premature breakdown of the stratum corneum. (C) Contact with external proteases such as detergents further increases the rate of rusting and desquamation. (D) This facilitates the influx of irritants and allergens through the skin to cause inflammation and infection. (8)

It is well established that the skin of AE patients tends to have higher TEWL and lower electrical resistance than normal controls suggesting (i) an impaired skin barrier, and (ii) that hydration of the SC is low, i.e. patients suffer from xerosis.(29, 45)

Treatments for AE depend on the severity of the disease and involve a combination of prophylactic approaches and therapeutic regimens tailored specifically for each individual. Generally, patients are advised to avoid contact with allergens and irritants, use soap substitutes, and regular use of emollients to alleviate dryness and increase the flexibility of the skin. The avoidance of certain foods which may trigger flare-ups may also be recommended.(46) However, the treatment of AE revolves around the use of topical corticosteroids (TCS) including hydrocortisone, betamethasone and fluticasone propionate.(1, 5, 47) They are normally used when emollient therapy is ineffective and may be used in conjunction with topical antibiotics such as mupirocin ointment.(46) TCS therapy may, however, only be used to control the manifestations and their use should be limited up to the point when improvement of the condition is seen, when the use of the drug should be gradually decreased or withdrawn.(48) The rationale is that the adverse effects caused by the long-term/overuse of these preparations cause local and systemic side-effects. The most prominent side-effect is skin thinning caused by impaired fibroblast proliferation induced by TCS. Skin atrophy decreases barrier function and increases the likelihood of further damage to the skin which may exacerbate the existing disorder.(49)



Fig. 7 Striae caused by topical corticosteroid use (50)

Local adverse effects include (i) atrophic changes - telangiectasia, striae (Fig. 7), ulceration and purpura, (ii) infections - granuloma gluteale infantum, masked microbial infections such as tinea incognito, (iii) steroid rebound or addiction, (iv) acne, and so on. Moreover, the systemic effects include hypothalamic-pituitary-adrenal (HPA) axis suppression, cataract, glaucoma, Cushing's syndrome, femoral head osteonecrosis and even, in rare cases, death due to corticosteroid-related Addison-crises.(47, 49, 51) In some cases, the

long-term use of potent and superpotent topical formulations have caused growth retardation in children.(51) For severe AE, immunosuppressive therapy involving topical calcineurin inhibitors - cyclosporin, tacrolimus and pimecrolimus - is used for controlling the disease. Although these drugs are very effective, concerns about their long-term use remain.(47, 48) Topical antihistamines are also prescribed for the treatment of AE. However, they can cause drowsiness.(1, 5, 47) Other treatments such as oral antibiotics (used to treat staphylococcal colonisation or infection of the skin) are also worth mentioning.(52) The vast majority of treatments for AE described above address the condition of the disease only after the manifestations have occurred and emollient therapy is only used to manage the problem of dry skin. The use of emollients is done on a trial and error basis and not all creams, ointments and lotions are suitable for each individual. Furthermore, this seemingly simple treatment is not free from problems. For example, the use of Aqueous Cream BP has been reported to cause burning, stinging and redness of the skin after application. In fact, the frequency of adverse reactions caused by Aqueous Cream BP is the highest amongst all emollients, yet it is the most widely prescribed formulation for the treatment of xerosis.(53) Aqueous Cream BP is used as an emollient to relieve skin dryness, improve flexibility and also serves as a mechanical barrier to irritants and allergens. Cutaneous reactions caused by Aqueous Cream BP could be due to two ingredients; the preservative phenoxyethanol and/or the surfactant sodium lauryl sulphate (SLS). SLS is widely used as a model skin irritant and is known to act as a penetration enhancer.(54) The use of SLS in formulations intended to treat AE defeats the object since it exacerbates the disease by enhancing the penetration of irritants and allergens that causes atopic manifestations. Recent research has shown that short term application of Aqueous cream BP on the forearm of healthy non-atopic volunteers induced skin atrophy (12%) and increased transepidermal water loss (20%) most likely the result of SLS contained in the preparation.(55) The use of this emollient clearly leads to negative effects on the stratum corneum and therefore, it is plausible to assume that this effect would be enhanced in AE sufferers and cause further damage to their delicate skin barrier, in which case the use of Aqueous Cream BP may benefit from a review.

In addition, as mentioned previously, the process of desquamation is finely maintained by a balance of skin proteases and their inhibitors. However, in AE, the up-regulation of these proteases, (chiefly SCCE and SCTE, and stratum corneum thiol protease (SCTP)) lead to increased desquamation causing the skin barrier to breakdown prematurely.(42, 43) Since

zinc is a known protease inhibitor, and can therefore counteract the effects of the proteases, it would be feasible to propose a zinc-containing formulation for topical application for treatment of AE to aid skin barrier repair.(42, 43)

Previous research carried out have shown that the activity of the two main proteases responsible for increased desquamation; kallikrein 5 and 7, which is known as stratum corneum tryptic enzyme (SCTE) and stratum corneum chymotryptic enzyme (SCCE), respectively, can be inhibited by zinc. The mechanism of action relies on the ability of zinc to bind to the active sites of the proteases to effectually 'deactivate' them. Once deactivated, premature desquamation will be decreased and the proteases can no longer breakdown corneodesmosomes in the stratum corneum in an uncontrolled manner.(42, 43) This reduction in the rate of desquamation will allow time for the proliferation and maturation of keratinocytes through the epidermis to eventually form the stratum corneum and the intercellular lipid lamellae which protects the body from influx of harmful substances. Increased rates of desquamation prevent the intercellular lipid lamellae from forming properly due to lack of time for the cell contents to be extruded into the intercellular space to be processed into the continuous lipid domain that keeps the skin intact.(33) Zinc is known to stabilise cell membranes and also reported to play a central role in cell mitosis, migration and maturation in dermal tissue.(56) Studies have also shown that zinc concentrations increase by approximately 15-20% in the areas around wounds within 24 hours. Further, it has been reported that topical application of zinc is more effective than oral zinc therapy at epithelialisation of wounds in normozincemic individuals and reducing infections.(56)

The primary objective of the project is to, therefore, formulate and safely deliver a topically applied drug to improve skin barrier function in AE and other related diseases. The aims of the project, therefore, are to (i) develop an *in vivo* methodology to characterise and assess skin barrier function, (ii) develop a method to extract and quantify the percutaneous uptake of zinc into the stratum corneum *in vitro*, (iii) determine the *in vitro* percutaneous penetration of zinc into the skin from commercial preparations, (iv) prepare and determine the *in vitro* percutaneous penetration of zinc into the skin from novel zinc formulations, (v) examine and image skin treated with various zinc preparations using scanning electron microscopy, and (vi) investigate the *in vivo* absorption of zinc from Sudocrem<sup>®</sup> and compare uptake using different extraction methods.



Should zinc be delivered efficiently and effectively to the skin, further development of the formulation into a marketable form may, in the long term, revolutionise the treatment of atopic eczema.



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**Chapter Two: Development of a Tool for the Evaluation  
of Healthy and Damaged Skin**



## **Chapter Two**

### **Development of a Tool for the Evaluation of Healthy and Damaged Skin**

#### **Summary**

*Background* - The development of a novel topical formulation for the treatment of atopic eczema is currently in progress. Like all products, subsequent to formulation, efficacy testing needs to be carried out. In order to do so, it will be necessary to establish a means to detect the formulation effects on the skin and, in this case, to be able to detect differences between healthy and diseased or damaged skin.

*Objectives* - To develop a tool that can suitably measure the physical properties of the skin to distinguish between healthy, intact skin and damaged or disease skin to provide an indication to the effects of a novel topical formulation.

*Methods* - Six human volunteers were recruited to apply Aqueous Cream BP to one side of their mid-volar forearms in an attempt to elicit a change in the skin barrier. The other side of the forearm was left untreated to act as a control. Tape stripping and transepidermal water loss measurements (TEWL) were used to characterise and assess the skin barrier function after treatment four weeks treatment.

*Results* - The data showed that the application of Aqueous Cream BP for four weeks was successful in causing a sufficient change in the skin barrier that was detectable by using the method developed to measure changes in the physical properties of the skin.

*Conclusions* - The tool developed using tape stripping and TEWL measurements in tandem for the characterisation and assessment of skin barrier function was suitably fit for purpose, reproducible, reliable and robust. The study also revealed that the short-term application of Aqueous Cream BP caused the barrier properties of the skin to diminish.

Keywords: Tape stripping, transepidermal water loss (TEWL), skin barrier, aqueous cream BP, sodium lauryl sulphate (SLS)



## **Introduction**

The intention of this study is to develop a suitable tool with which to assess and characterise the skin barrier. Its necessity originates from the current development of a novel formulation for the treatment of skin disorders such as eczema where patients are afflicted with an abnormal skin barrier. This defect in the protective layer, the stratum corneum, allows higher levels of transdermal water loss to occur from the surface of the skin.(1) More alarmingly, this also permits the influx of irritants, chemicals and xenobiotics through the barrier which eventually leads to flare-ups of eczematous lesions. The formulation is anticipated to aid repair of the skin barrier in the hope of reducing the frequency of flare-ups experienced. Therefore, to be able to determine the effectiveness of the formulation, a tool needs to be developed that is able to detect differences in the skin barrier before and after treatment, i.e. a damaged and healthy, healed skin barrier.

The concept of this tool is based on Fick's 1<sup>st</sup> Law of Diffusion which states that the passive transport of water molecules across membranes progress from regions of high concentration to regions of low concentration, i.e. mass transfer.(2)

$$J = \frac{K \cdot \Delta C \cdot D}{H} \quad (Eq.1)$$

This movement of water across membranes can be explained via the above equation where flux, J, (which, in this case is water) it is equal to the product of the SC-viable epidermis partition coefficient of water, K (0.06), multiplied by the concentration gradient of water across the stratum corneum,  $\Delta C$  ( $\sim 1\text{g/cm}^{-3}$ ), multiplied by the diffusivity of water within the stratum corneum, D, divided by the diffusional pathlength, H.(2,3) In simple terms, flux is inversely proportional to stratum corneum thickness and by using tape stripping, we can determine the thickness of the barrier (Eq. 1). This information can be used to indicate the condition of the skin barrier since a diminished stratum corneum, in theory, should allow more water to pass freely from the surface of the skin.

A modified version of Fick's 1<sup>st</sup> Law of Diffusion allows the flux at various depths of the stratum corneum layers to be measured during tape stripping and equation 2 has, incorporated into it,  $J_x$ , the value of TEWL at a depth of  $x$  within the stratum corneum and,  $x$ , the thickness of stratum corneum removed.(1)

$$J_x = \frac{K \cdot D \cdot \Delta C}{(H - x)} \quad (Eq.2)$$

Since we know that the stratum corneum forms the rate-limiting barrier to insensible water loss and from the equations we also know that TEWL is inversely proportional to stratum corneum thickness we can use these parameters to form the core of the tool that will be used to assess and characterise skin barrier function and differentiate between healthy and damaged skin barrier.

## **Materials and Methods**

### **Materials**

Aqueous Cream BP (cetostearyl alcohol, sodium lauryl sulphate, liquid paraffin, white soft paraffin, phenoxyethanol 1% w/w and purified water) was purchased from Pinewood Healthcare (Clonmel, Ireland). Scotch No. 845 book tape was from 3M (St. Paul, MN, USA), Micropore<sup>TM</sup> tape from 3M (Nadarzyn, Poland), and the tape stripping template (2 cm diameter aperture) was constructed from the previously mentioned book tape.

### **Study Population**

Six healthy female subjects aged between 20-36 years with no history of skin disease, and who did not suffer from any dermatological conditions at the time of the study participated in the investigation. Each person was provided with an information document containing the project details. Written informed consent was obtained from each participant and any questions raised were answered appropriately and duly. The study was approved by the local research ethics committee (Royal United Hospital, Bath, UK).

Throughout the duration of the study, participants were required to refrain from applying any topical formulations to the test areas, i.e. forearms, and to avoid taking part in any

activities that may affect the properties of the skin such as sunbathing, swimming, exfoliation and beauty treatments. In addition, participants were also instructed to allow a grace period of 2-3 hours before engaging in activities such as taking a bath/shower or sporting events after the application of Aqueous Cream BP.

### Experimental Procedure - Treatment with Aqueous Cream BP

The experiments were conducted on the mid-volar forearm region with both control and test areas in close propinquity to each other (without overlapping) to minimise intra-individual and regional differences in the skin barrier. To partition the test and control regions of the skin a hypothetical line was envisaged from wrist to elbow with each region containing two test sites. 2 ml of Aqueous Cream BP was delivered to the test area using a syringe (without the needle) and massaged uniformly. The formulation remained on the area for ten minutes after which the excess was removed using soft facial tissue, taking care not to contaminate the contralateral (control) region. The application was carried out bi-daily (morning and night) for a period of four weeks. Volunteers were asked to conduct the last application 24 hours prior to tape-stripping. The procedure was also conducted on the opposite arm where the test and control regions were reversed (Fig. 1)

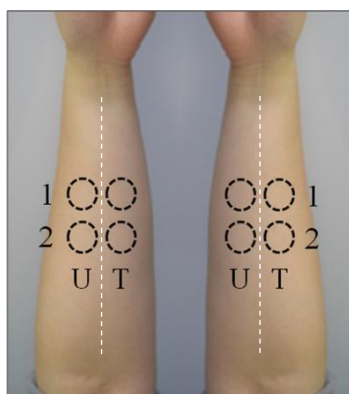


Fig. 1 Illustration of the treated and control regions of the mid-volar forearm where the treatment sites are reversed for opposite arms. The lines delineate the boundary between treated and untreated sites.

### Tape Stripping

Volunteers were required to acclimatise to the laboratory environment for at least 20 minutes before commencing the experiment. To delineate the tape-stripping area, a polyester template with a circular aperture of 2 cm diameter was attached to the mid-volar forearm. Baseline TEWL was measured using a closed-chamber evaporimeter (Biox Aquaflux AF102, Biox Systems Ltd. London, UK). Multidirectional tape stripping with

pre-weighed tape strips (Sartorius AG SE-2F semi-microbalance, Sartorius AG, Göttingen, Germany, 0.1 µg precision) and TEWL measurements were carried out until approximately 75% SC removal was achieved. The tape strips, with skin adhered, were then re-weighed to allow stratum corneum mass and hence, thickness to be calculated. To ensure uniform adhesion between the tape and skin, systematic application of homogeneous pressure using a weighted roller was employed.

### Data and Statistical Analysis

SC thickness removed was calculated using the following equation where SC density is assumed to be 1 g cm<sup>-3</sup>: (4)

$$\text{SC thickness removed} = \frac{\text{mass of SC removed}}{\text{area of SC removed} \times \text{SC density}} \quad (\text{Eq.2})$$

Preliminary data analysis was carried out using Fick's 1<sup>st</sup> Law of Diffusion as explained previously (Eq. 3):(2)

$$J_x = \frac{K \cdot D \cdot \Delta C}{(H - x)} \quad (\text{Eq.3})$$

A linear relationship derived from the above equation allows for the total thickness of the stratum corneum to be determined (Eq 4):(2)

$$\frac{1}{\text{TEWL}_x} = \frac{H}{K \cdot D \cdot \Delta C} - \left[ \frac{1}{K \cdot D \cdot \Delta C} \right] x \quad (\text{Eq.4})$$

such that plotting 1/TEWL<sub>x</sub> as a function of cumulative thickness removed (x) yields a straight line, the x-intercept of which equals the total SC thickness, H (Fig 2).

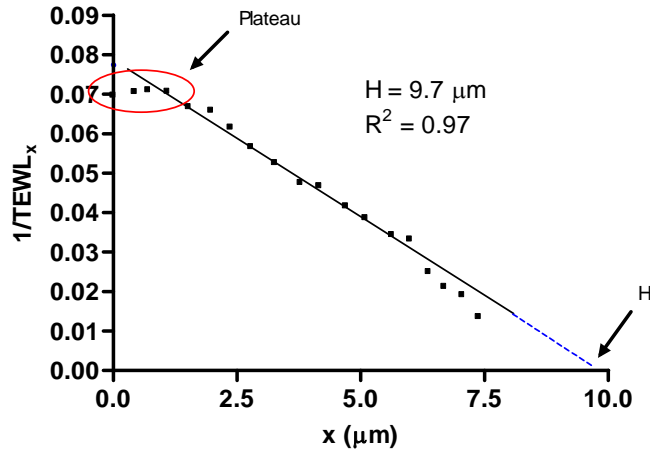


Fig. 2 Example graph of  $1/TEWL_x$  versus SC thickness removed for one volunteer. A linear fit of Fick's 1<sup>st</sup> Law of Diffusion allows the total SC thickness to be deduced from the x-intercept.

However, quite often, it is apparent that the initial values of TEWL do not change significantly as the first tape strips are removed (Fig. 2). This may be due to the stratum disjunctum which is not contributing to the overall barrier function. The result is that the effective value of H may be over-estimated. To circumvent this problem, the results have been analysed with a modified model, recently published by Russell and Delgado-Charro (2008).(5)

$$Y = B + \frac{K \cdot D \cdot \Delta C}{(H - x)} \quad (Eq.5)$$

where B is the baseline TEWL and  $K \cdot D \cdot \Delta C$  is a constant (30). TEWL values as a fraction of x were fitted this equation which takes all points into consideration and enhances the accuracy of the SC thickness determination (Fig. 3).

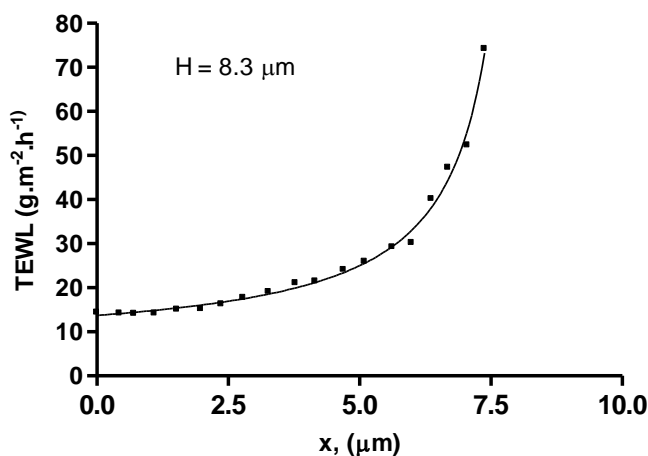


Fig. 3 Re-plotted data from Fig 2 fitted to the modified model of Russell and Delgado-Charro (2008). For the fitting procedure, initial values of B, P and H were  $14.4 \text{ gm}^{-2}\text{h}^{-1}$ , 30 units, and  $8.4 \text{ }\mu\text{m}$ .

GraphPad Prism V.4.00 (GraphPad Prism<sup>®</sup> 4 Software, San Diego, CA, USA) was used to calculate stratum corneum thickness via non-linear regression and also all statistical calculations. Stratum corneum thickness and TEWL values were expressed in terms of standard deviations (SD) and means. Confirmation of Gaussian distribution was established by performing a normality test on the data and statistical significance was determined via a two-tailed paired t-test. The confidence interval was adjusted to 95% and the significance level was set at  $P < 0.05$ .

## **Results**

### **Tape Stripping Profile**

A typical tape stripping profile of treated and untreated skin is displayed in Fig. 4. After four weeks treatment with SLS containing Aqueous Cream BP a change in the skin barrier can be detected using TEWL and tape stripping. The graph shows an increase in the baseline TEWL and a faster rate of increase in TEWL during tape stripping. In addition, a decrease in stratum corneum thickness can be observed in the treated skin.

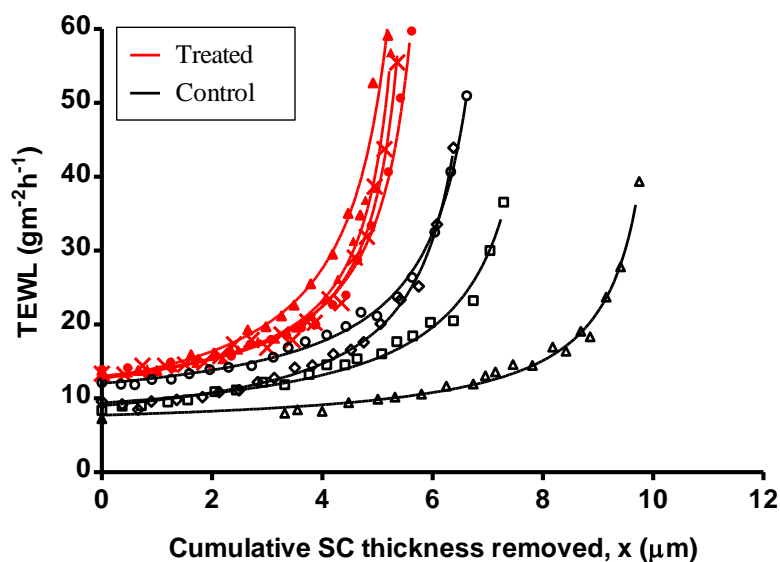


Fig. 4 Representation of the changes in the tape stripping profile of treated and untreated skin

#### Total Stratum Corneum Thickness and TEWL

On closer inspection of the data, we can see an inversely proportional relationship between total stratum corneum thickness and TEWL, as predicted by Fick's 1<sup>st</sup> Law of diffusion, between the pairs of data (Table 1). (1) Total stratum corneum thicknesses of control and treated skin sites ranged from 6.0 - 15.3  $\mu\text{m}$  (mean  $9.3 \pm 2.5\mu\text{m}$ ) and 4.5 - 14.1  $\mu\text{m}$  (mean  $8.2 \pm 2.5 \mu\text{m}$ ), respectively. For TEWL, control and treated baseline values ranged from 7.2-22.0  $\text{gm}^{-2}\text{h}^{-1}$  (mean  $12.3 \pm 3.6 \text{ g.m}^{-2}\text{h}^{-1}$ ) and 11.2 - 22.0  $\text{g.m}^{-2}\text{h}^{-1}$  (mean  $14.8 \pm 2.4 \text{ g.m}^{-2}\text{h}^{-1}$ ), respectively. An overall decrease of 1.1  $\mu\text{m}$  in stratum corneum thickness and an increase of 2.5  $\text{gm}^{-2}\text{h}^{-1}$  in TEWL was observed.

Table 1 Differences between treated and untreated total SC thickness and TEWL are small but significant. An increase in SC thickness and decrease in TEWL after Aqueous Cream BP application is seen in the majority of cases

Subject	Control H (μm)	Treated H (μm)	Δ H (μm)	% Δ H (μm)	Control baseline TEWL (g·m <sup>-2</sup> ·h <sup>-1</sup> )	Treated baseline TEWL (g·m <sup>-2</sup> ·h <sup>-1</sup> )	Δ TEWL (g·m <sup>-2</sup> ·h <sup>-1</sup> )	% Δ TEWL (g·m <sup>-2</sup> ·h <sup>-1</sup> )
1	11.5	9.8	-1.6	-14	10.3	11.7	1.4	14
1	8.8	10.6	1.8	20	11.3	13.7	2.4	21
1	8.9	8.3	-0.6	-6	15.9	14.4	-1.5	-10
1	9.2	9.0	-0.2	-2	12.5	15.2	2.7	22
2	8.5	6.0	-2.5	-29	8.4	13.8	5.4	64
2	10.5	5.8	-4.7	-45	7.2	13.4	6.2	85
2	7.5	6.3	-1.2	-16	12.1	13.9	1.8	15
2	7.2	5.9	-1.3	-18	9.6	13.3	3.8	39
3	6.3	6.6	0.3	5	12.0	13.5	1.5	12
3	9.6	6.7	-2.8	-30	8.5	11.8	3.3	39
3	9.7	9.3	-0.4	-4	7.9	12.2	4.4	56
3	11.0	9.3	-1.6	-15	7.4	12.1	4.7	64
4	12.9	14.1	1.2	10	11.7	15.6	3.8	33
4	13.1	13.4	0.4	3	10.6	14.9	4.3	41
4	14.8	11.6	-3.2	-21	11.9	15.4	3.5	29
4	15.3	10.9	-4.3	-28	12.2	14.3	2.2	18
5	9.4	9.2	-0.2	-2	12.2	13.3	1.0	8
5	10.0	9.1	-0.9	-9	10.8	11.2	0.4	4
5	8.6	9.5	0.9	10	11.8	15.9	4.1	34
5	9.6	9.7	0.1	1	10.9	17.5	6.7	62
6	6.0	4.5	-1.4	-24	17.0	17.7	0.6	4
6	6.5	5.7	-0.9	-13	18.3	16.1	-2.2	-12
6	7.0	7.2	0.2	2	13.7	16.1	2.3	17
6	7.5	7.1	-0.3	-5	12.1	15.1	3.0	24
6	8.4	6.0	-2.4	-29	15.7	19.2	3.5	22
6	7.1	5.8	-1.3	-18	18.5	17.2	-1.3	-7
6	6.5	5.0	-1.5	-23	22.0	22.0	0.0	0
Mean ± SD	9.3 ± 2.5	8.2 ± 2.5	-1.1*	-11.8	12.3 ± 3.6	14.8 ± 2.4	2.5 **	+20.8

Fig. 5 shows the data in a before- and after- graph. It clearly illustrates the changes in total stratum corneum thickness and TEWL after Aqueous Cream BP application (Fig. 5). In 20 of 27 cases (74% of skin sites), a decrease in total stratum corneum thicknesses was observed post treatment with a mean decrease of 1.7 μm, a percentage change of 18 % ( $P < 0.0001$ ). Overall, where  $n = 27$ , total stratum corneum thickness decreased, by 1.1 μm, which translates to a percentage decrease of 12% ( $P = 0.0015$ ).

For baseline TEWL, a mean increase of 3.2 g·m<sup>-2</sup>·h<sup>-1</sup>, correlating to a percentage increase of 29%, was observed in 23 out of 27 cases (85% of skin sites) after Aqueous Cream BP application ( $P < 0.0001$ ). Overall, an increase of 2.5 g·m<sup>-2</sup>·h<sup>-1</sup>, a percentage increase of 20%, was observed across the board ( $P < 0.0001$ ). Only one pair of values showed no increase or decrease in TEWL after treatment with the cream.



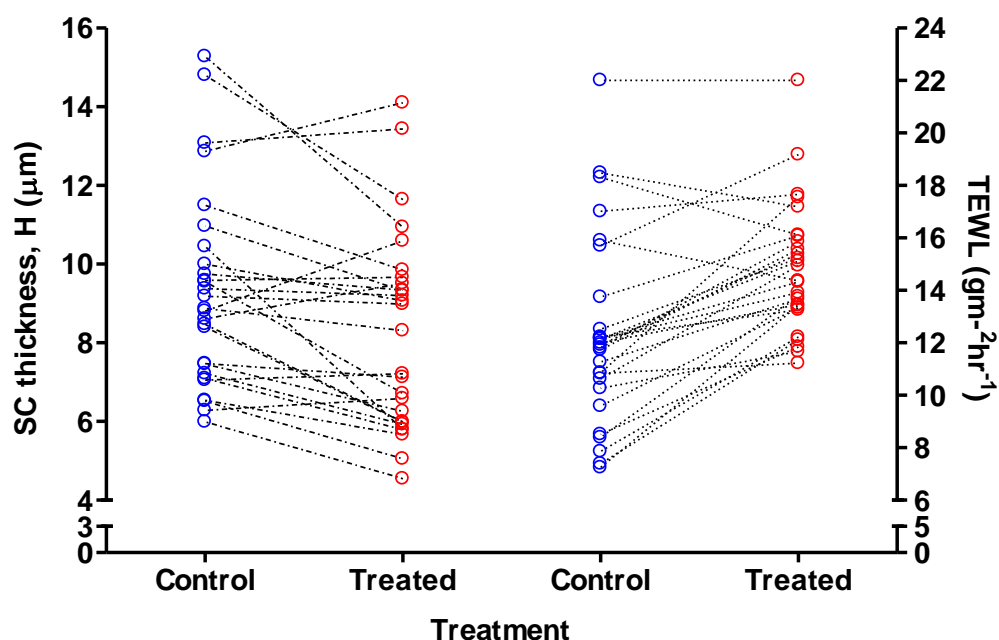


Fig. 5 Graphical representation of changes in total stratum corneum thickness and TEWL

#### Fick's 1<sup>st</sup> Law of Diffusion

Out of 27 pairs skin sites sampled, 16 of those followed Fick's 1<sup>st</sup> Law to demonstrate that a decrease in stratum corneum thickness is accompanied by an increase in TEWL. Fig. 6 permits different combinations of increases and decreases in changes of stratum corneum thickness and TEWL to be visualised. Increased thickness with increased TEWL, increased thickness with decrease TEWL, and decreased thickness with decreased TEWL, and most importantly, decreased thickness accompanied by increased TEWL are located in the upper right quadrant (green), lower right quadrant, lower left quadrant (blue) and upper left quadrant (red), respectively. The graph shows that the majority 16 out of 27 (56%) of the data points are positioned in the upper left quadrant indicating a decrease in stratum thickness and an increase in TEWL

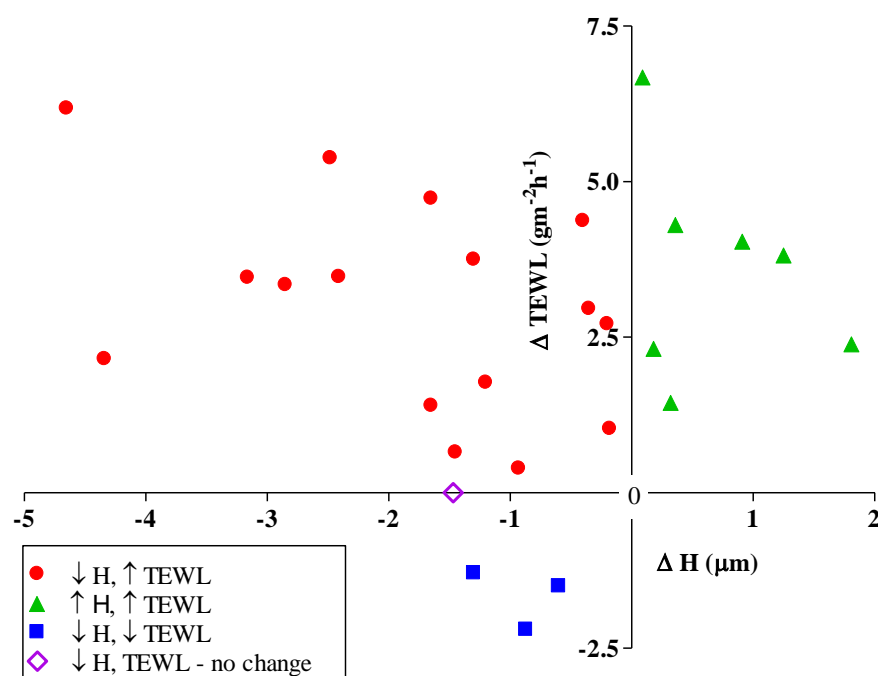


Fig. 6 A scatter plot illustrating different combinations of changes in stratum corneum thickness and TEWL

## Discussion

In the first part of this study, healthy human volunteers with no history of dermatological disease were asked to apply Aqueous Cream BP to their forearms bi-daily for four weeks in an attempt to perturb the skin barrier. The formulation contains approximately 1 % w/w of sodium lauryl sulphate (SLS). SLS is an anionic surfactant that is known to cause irritation to the skin and is routinely used to alter and disturb the skin barrier in percutaneous penetration, barrier function and barrier repair studies.(1, 6-18) SLS is also extensively used to form disease models such as irritant contact dermatitis in studies and for the investigation of skin barrier properties in atopic dermatitis sufferers.(13-15) The concentrations used in these studies ranged from 0.03% - 20% w/v and with the exception of Koopman *et al*, all researchers reported a change in the skin barrier which was measured as an increase in TEWL and/or visual score of erythema.(6-18) In accordance with previous research using concentrations close to, or lower than, the 1% w/v used in this study, the application of SLS also resulted in an increase in TEWL.(8, 11, 13, 14, 17, 18) The effectiveness of SLS as a barrier irritant is demonstrated by the use of a 1% w/v

solution for just 20 minutes resulting in barrier perturbation presented as increase in TEWL.(17)

In our study, SLS damaged skin was compared to unperturbed healthy skin sites in order to test the efficiency of the TEWL and tape stripping as tools for detecting changes in skin barrier function. The application of Fick's 1<sup>st</sup> Law of Diffusion involved the use of these two techniques that formed the second part of the experiment. These two techniques are well established and frequently used in the area of skin research.(1, 2, 5, 6, 8, 10, 18) TEWL is the non-invasive measurement of the water-vapour gradient between the skin surface and the external environment.(19) It is measured by placing an evaporimeter probe onto the surface of the skin until a stable reading is recorded and, when used exclusively, it can detect changes in the skin barrier even in visually intact skin.(18)

TEWL is impeded by the intercellular lipid pathway of the stratum corneum, which forms the rate-limiting barrier to insensible water loss, and any damage or removal of this layer by tape stripping or the removal or disruption of intercellular lipids decreases the tortuous pathway of water to the external environment resulting in TEWL increase.(1, 9-18, 20-22) Hence, TEWL can be used as a diagnostic tool to assess damage to the skin barrier and since Fick's 1<sup>st</sup> Law of Diffusion states that the flux (of water) is inversely proportional to stratum corneum thickness, tape stripping was used in conjunction to form the core of our tool.(1)

Tape stripping is a procedure that uses adhesive tape to successively remove layers of the stratum corneum.(2, 20-22) It is often used to quantify the amount of substances absorbed on or into and through the skin. Drug bioavailability, bioequivalence and drug penetration in the stratum corneum can be determined using this simple method.(6, 18, 23-26) Barrier recovery studies and stratum corneum thickness information can also be obtained in this manner.(2, 10-12, 20-22) A more recent study looks at the application of quantum dots to the skin, using tape stripping and transmission electron microscopy to assess penetration.(27)

In this investigation, tape stripping was used to determine stratum corneum thickness and, used in affiliation with TEWL, a tape stripping profile was obtained which was used to study the condition of the skin barrier (Fig. 4). A normal healthy skin barrier is approximately 10-20  $\mu\text{m}$  thick and has a TEWL of about 10-12  $\text{gm}^{-2}\text{h}^{-1}$ .(19) In this study,

mean stratum corneum thickness found in untreated skin of volunteers was slightly lower (9.3  $\mu\text{m}$ ) than previously reported by Schaefer and Redelmeier (19) and after treatment with Aqueous Cream BP, the mean value was reduced by 12% to 8.2  $\mu\text{m}$ . Baseline TEWL before treatment was slightly higher at 12.3  $\text{gm}^{-2}\text{h}^{-1}$ , than the range mentioned previously, and increased to 14.8  $\text{gm}^{-2}\text{h}^{-1}$  after treatment. The minute change in stratum corneum thickness by 1.1  $\mu\text{m}$  and TEWL by 2.5  $\text{gm}^{-2}\text{h}^{-1}$  was detected by tape stripping and evaporimeter. This demonstrates that both methods are suitably sensitive for our purposes of detecting changes in skin barrier function.

### **Conclusion**

TEWL and tape stripping used in combination provides a powerful and yet simple and efficient minimally-invasive method for the characterisation and assessment of skin barrier function both *in vitro* and *in vivo* and is suitable for the purpose, in our investigations, for studying the active ingredient penetration profile of topical formulations and also for determination of skin barrier function.

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**Chapter Three: Delivery of Zinc to the Skin - Establishment  
of an Analytical Method**





## **Chapter Three**

### **Delivery of Zinc to the Skin - Establishment of an Analytical Method**

#### **Summary**

*Background* - There are many zinc containing formulations for the treatment of skin conditions available on the market today. To formulate a novel zinc containing topical product for the treatment of eczema, it is first necessary to investigate the ability of zinc to penetrate into the skin and, second, an analytical technique that can quantify the amount delivered needs to be established.

*Objectives* - To assess the *in vitro* delivery of zinc into the skin by passive diffusion from aqueous solutions of zinc chloride ( $\text{ZnCl}_2$ ) and to develop a methodology to quantify the amount penetrated into the stratum corneum.

*Method* - Aqueous solutions of 5% and 50% w/v zinc chloride ( $\text{ZnCl}_2$ ) were delivered to porcine abdominal skin by passive diffusion for six hours. The skin was tape stripped and the zinc was extracted using 10% acetic acid. Analysis was carried out using atomic absorption spectroscopy (AAS).

*Results* - The amounts of zinc delivered to the skin from 5% and 50% w/v  $\text{ZnCl}_2$  solutions were  $57 \pm 21 \mu\text{g}$  and  $489 \pm 117 \mu\text{g}$  ( $P < 0.0001$ ), respectively. No penetration of zinc was observed through the skin into the receptor solution.

*Conclusions* - Passive diffusion of zinc into the SC was ten-fold greater from 50% w/v  $\text{ZnCl}_2$  compared to a 5% w/v solution. There was negligible transport of zinc across the skin, however, from either solution. AAS proved sufficiently specific, sensitive and reliable to assess the performance of zinc containing formulations.

Keywords: Tape stripping, zinc chloride, passive diffusion, atomic absorption spectroscopy (AAS), zinc

## **Introduction**

Having established a method to characterise and assess the skin barrier function both *in vitro* and *in vivo*, as previously described in Chapter 2, the next step leading to the production of a novel zinc containing formulation is the investigation of zinc delivery to the skin *in vitro* and establishing an analytical method to assess and quantify the amount of zinc extracted from skin samples.

There are a multitude of commercial zinc oxide formulations available on the market today.(1) The majority comprise creams or ointments which act as physical barriers to protect the skin from contact with irritating substances such as urine in the case of nappy rash, and also to prevent the increased loss of water through damaged skin in conditions such as eczema and psoriasis.(2-4) In addition to topical preparations, medicated bandages containing zinc paste are also available and are used to treat lichenification in psoriasis and chronic eczema due to repeated scratching.(1, 5) The formulations contain zinc as the active ingredient mostly in the form of zinc oxide with concentrations ranging from 7.5 - 38 % w/w dispersed in a mixture of waxes, fats and other supposedly beneficial substances such as ichthammol, cod-liver or castor oil.(1, 4, 6) However, literature regarding these formulations, their efficacy or the penetration of zinc into the skin is scarce. Sunscreens represent another, important category of zinc containing formulations used for non-medicinal purposes.(1, 4) With the exception of sunscreens, the efficiency of zinc delivery from these medicinal formulations for the treatment of skin conditions has not been studied in depth.

Zinc is an essential mineral in the body and is a co-factor of more than 300 metalloenzymes (including alcohol dehydrogenase, angiotensin converting-enzyme and alkaline phosphatase) and in excess of 2000 transcription factors. Zinc is acknowledged to be essential for the normal development and physiology of humans as it is involved in the synthesis, transcription and translation of RNA and DNA.(7-9) After iron, zinc is the second most abundant trace element in the body.(9) An average human adult of 65kg (of which 7kg is skin) contains about 2.5g zinc, 6% of which is present in the skin.(10, 11) The significance of zinc to dermatological health is apparent when, in situations where a deficiency occurs due to dietary insufficiency or genetically induced malabsorption from the gastrointestinal tract, skin lesions in the form of eczema, impaired wound healing,

epidermolysis bullosa simplex and epidermolytic hyperkeratosis (which causes blistering of the skin) occur.(12, 13) Zinc was recognised more than 3000 years ago by the ancient Indians and used in Ayurvedic medicine as documented in the Papyrus Ebers as beneficial for the skin and was used as a topical treatment for wound healing in the form of zinc oxide or calamine.(8, 9) In conditions such as atopic eczema, where premature and enhanced desquamation occurs, the incorporation of zinc into a formulation for topical application would neutralise excess proteases and aid restoration of the skin barrier.(14, 15)

The amount of zinc present in the skin is a testament to its importance to skin health and barrier function since it is involved in morphogenesis, and more relevant to this thesis, repair and maintenance of the skin barrier.(11) Where a diet is deficient in this mineral, dermatological symptoms such as periorificial and acral skin lesions in acrodermatitis enteropathica, skin ulcers, xerosis, roughness of the skin, eczema and delayed wound healing occur.(8, 10, 12) The importance of zinc to the condition and health of skin is evident in (for example) psoriasiform dermatitis (caused by poor absorption of zinc in acrodermatitis enteropathica) and chronic leg ulcers, conditions which are successfully treated with oral zinc supplements and topical zinc oxide preparations, respectively.(8) Furthermore, research has shown that zinc accumulates in wounds inflicted on the back of mice indicating its role in repair and healing and the use of zinc supplements increase healing rates in damaged tissues.(11, 16) Zinc deficient mice also display delayed wound epithelialisation.(11) Another example of the importance of zinc to skin health is demonstrated by the apoptosis of human keratinocytes when placed in zinc-deficient medium.(13) It is obvious that zinc plays a major role in the health of the skin, however; it is well documented that zinc penetration into the skin is limited.(17-21). Since the development of a novel zinc containing formulation for the treatment of eczema is currently in progress, the penetration of zinc into the skin and an analytical technique for its quantification need first to be established.

In this study, an investigation assessing the percutaneous penetration of zinc *in vitro* was performed using dermatomed abdominal porcine skin in Franz diffusion cells. Zinc delivery into the stratum corneum (SC), and across the skin, from 5% and 50% w/v aqueous zinc chloride solutions was determined using tape stripping after a six hour

treatment and analysis of the samples, post-extraction, was carried out by atomic absorption spectroscopy (AAS).

### **Materials and Methods**

#### Materials

Chemicals: Zinc chloride, 99.995 +% purity was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Sodium chloride, disodium hydrogen phosphate, potassium phosphate, and potassium chloride were purchased from Acros Organics (Geel, Belgium). Zinc nitrate standard solution was from SpectroSol<sup>®</sup> (BDH Chemicals Ltd., Poole, UK).

#### Skin preparation

Porcine skin obtained from a local piggery (B & J Pigs Ltd., Somerset, UK) was cleaned under cold running water and dermatomed to a thickness of approximately 750  $\mu\text{m}$  (Zimmer<sup>™</sup> Electric Dermatome, Dover, OH, USA). The pieces of skin were then wrapped in Parafilm<sup>™</sup>, placed into a sealed bag and stored in a freezer at  $-20^{\circ}\text{C}$  until required.

#### *In vitro* passive diffusion experiments

A piece of frozen porcine abdominal skin was thoroughly thawed and visually inspected for defects and punctures. (Previous studies have shown pig skin to be a well-established model for human skin for both *in vivo* and *in vitro* studies and that storage at  $-20^{\circ}\text{C}$  generally does not alter percutaneous absorption.)(22, 23) Excess hair was carefully trimmed using scissors and the skin was divided into three pieces; control, treated with 5% w/v aqueous  $\text{ZnCl}_2$ , and treated with 50% w/v aqueous  $\text{ZnCl}_2$ . The pieces of skin were mounted into vertical Franz diffusion cells (PermeGear, Inc., Bethlehem, PA, USA) with the external surface, the stratum corneum side, facing the donor chamber (donor volume = 3 ml). The area available for transport was approximately  $3\text{ cm}^2$ . The receptor chambers (volume = 7 ml) were filled with phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl) at pH 7.4. To verify skin integrity and allow thermal equilibration, the cells were inverted and observed for leaks. After 30 minutes the cells were returned to the upright position and connected to a syringe pump (“Genie” Kent programmable syringe pump, Kent Scientific Corporation, Litchfield, CT, USA). Timing of the experiments commenced when the donor chambers were filled with their respective

donor solutions and covered with Parafilm™. The receptor solutions were stirred magnetically throughout the experiment. Passive diffusion took place over 6 hours at room temperature and 1 ml samples of the receptor solution were collected every hour. For every 1 ml collected, an equal volume of fresh PBS was delivered by the syringe pump. At the end of the experiment, the donor solutions were collected and reserved for analysis. Between collection and analysis, the samples were stored at 4°C. The pieces of skin were dismantled from the cells and thoroughly dried using lint-free absorbent tissue before being temporarily stored between sheets of Parafilm™ until the tape stripping procedure. A minimum of ten replicates per donor solution was performed and the skin from five different pigs was used in the experiments.

#### Tape-stripping

Endogenous zinc (control) and the amounts delivered into the stratum corneum were assessed following tape-stripping. After drying, the skin was pinned to a polystyrene board and a template with a circular aperture (diameter = 2 cm) was affixed to delineate the area to be sampled. Baseline transepidermal water loss (TEWL) was measured (Biox Aquaflux AF102, Biox Systems Ltd., London, UK) and the skin was subjected to multidirectional sequential tape stripping with adhesive tapes (Scotch No. 845 book tape. 3M, St. Paul, MN, USA) that had been previously weighed using a 0.1 µg precision balance (Sartorius AG SE-2F semi-microbalance, Sartorius AG, Göttingen, Germany). TEWL measurements were made after every other tape strip and monitored as an indicator of total SC removal (when TEWL ~100 g.m<sup>-2</sup>h<sup>-1</sup>).

#### Sample Analysis

After tape-stripping, the tapes were re-weighed to allow the mass and thickness of SC removed to be calculated. The zinc was extracted from the tapes by shaking overnight (IKA HS 260 Basic shaker, IKA® Werke GmbH & Co., KG, Germany) in 10% acetic acid. Prior to analysis, all samples were appropriately diluted with 10% acetic acid to facilitate analysis by atomic absorption spectrometry (AAS) (Varian AA-275, Varian, Inc., Oxford, UK). The instrument was calibrated using 0.5, 1.0 and 2.0 ppm zinc nitrate calibration standards and the samples were analysed at 213.9 nm with a spectral bandwidth of 1 nm.

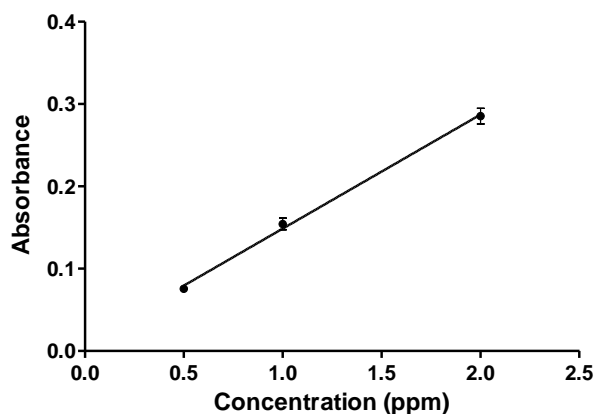


Fig. 1 Calibration curve for zinc standards using the AAS. Readings were obtained in triplicate. ( $r^2 = 0.9935$ )

To enable an estimation of mass balance, the original donor solutions of 5% w/v and 50% w/v aqueous  $\text{ZnCl}_2$  (prior to passive diffusion experiments) were also diluted and analysed. All solutions were prepared with deionised water, 18.2 M $\Omega$ -cm (NANOpure<sup>®</sup> DIamond<sup>™</sup> Life Science (UV/UF) Ultrapure Water System, Barnstead International, Dubuque, Iowa, USA).

### Data Analysis and Statistics

SC thicknesses were calculated from the TEWL measurements recorded during tape stripping using a modified form of Fick's 1<sup>st</sup> Law of diffusion (*Eqn. 1*).<sup>(24)</sup>

$$J = \frac{K \cdot \Delta C \cdot D}{H} \quad (\text{Eqn. 1})$$

Because TEWL changes only very slightly (if at all) during the removal of the first one or two tape strips, a baseline-corrected, non-linear model (*Eqn. 2*) has been proposed to fit TEWL data recorded during SC tape stripping.<sup>(25)</sup>

$$\text{TEWL}_x = B + \frac{K \cdot D \cdot \Delta C}{H - x} \quad (\text{Eqn. 2})$$

Where  $TEWL_x$  represents the flux of water across the stratum corneum the thickness of which has been reduced from  $H$  to  $(H - x)$  by stripping,  $K$  is the stratum corneum -viable epidermis partition coefficient of water (0.06),  $D$  is the diffusivity of water within the SC,  $\Delta C$  is the concentration gradient of water across the stratum corneum ( $\sim 1\text{g}/\text{cm}^{-3}$ ), and  $H$  is the total stratum corneum thickness. (Eqn. 2).(24, 26) In this equation,  $K \cdot D \cdot \Delta C$  is assumed constant (30) for any volunteer at any time.(25) This method enables all data to be taken into account and the stratum corneum thickness is calculated using non-linear regression (Fig. 2). In order to be able to make comparisons between individual pieces of skin, which have different stratum corneum thickness of course, all data were evaluated as a function of normalised position within the stratum corneum as previously reported and discussed in detail (24, 25).

Normality tests, non-linear regression and statistical analyses were performed using GraphPad Prism<sup>®</sup> 4.00 software (Graph Pad Software Inc., San Diego, CA, USA). Statistical significance between multiple data sets was determined via one-way ANOVA. The confidence interval was adjusted to 95% and the significance level was set at  $P < 0.05$ . Outliers, determined by Grubb's test (<http://www.graphpad.com/quickcalcs/index.cfm>), were removed from data analysis.

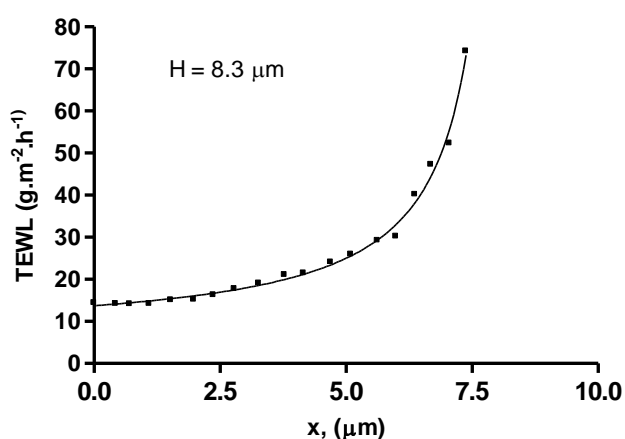


Fig. 2 An illustrative graph showing non-linear regression of the change in TEWL as a function of the amount of stratum corneum removed using the modified form of Fick's 1<sup>st</sup> Law of diffusion (25) . In this example, for the fitting procedure, initial values of  $B$ ,  $K \cdot D \cdot \Delta C$ , and  $H$  were  $14.4\text{ g m}^{-2}\text{ h}^{-1}$ ,  $30\text{ g cm}^{-1}\text{ h}^{-1}$ , and  $7.4\text{ }\mu\text{m}$ , respectively. The values for best fits for  $B$ ,  $K \cdot D \cdot \Delta C$ , and  $H$  were calculated to be  $6.3\text{ g m}^{-2}\text{ h}^{-1}$ ,  $61.8$  and  $8.3\text{ }\mu\text{m}$ , respectively.



## Results

The change in TEWL as a function of tape-stripping from the skin samples that were used for the control and zinc uptake experiments was quite consistent. The fitted values of  $B$ ,  $K \cdot D \cdot \Delta C$  and  $H$  (according to *Eqn. 2*) are in Table 1, and reveal no significant differences between any of the derived parameters.

Table 1 Fitted values (mean  $\pm$  SD;  $n = 10$  for controls and  $n = 11$  for 5% and 50%  $\text{ZnCl}_2$ ) of  $B$ ,  $K \cdot D \cdot \Delta C$  and  $H$  from measurements of TEWL as a function of tape-stripping

Treatment	Baseline TEWL correction value, $B$	$K \cdot D \cdot \Delta C$ , ( $\text{g cm}^{-1} \text{h}^{-1}$ )	Total SC thickness, $H$ ( $\mu\text{m}$ )
Control	$5.1 \pm 9.0$	$164 \pm 87$	$12.4 \pm 4.2$
5% $\text{ZnCl}_2$	$-4.0 \pm 12.5$	$287 \pm 198$	$13.4 \pm 4.1$
50% $\text{ZnCl}_2$	$5.8 \pm 5.3$	$87 \pm 61$	$13.7 \pm 1.9$

Total zinc recoveries for control, 5% and 50% w/v  $\text{ZnCl}_2$  treatments were  $2.8 \pm 0.69 \mu\text{g}$ ,  $45.2 \pm 4.05 \text{ mg}$ , and  $385 \pm 60.6 \text{ mg}$ , respectively (Table 2). The latter two values correspond to 85% of the applied amounts. Apparent losses of approximately 15% were most likely the result of residual solution on glassware and that remaining on the surface of the skin which was removed by wiping prior to tape stripping.

Table 2 Zinc uptake results into (mean  $\pm$  SD;  $n = 10$  for controls,  $n = 11$  for zinc chloride treatments and through skin after treatment with either 5% or 50% w/v  $\text{ZnCl}_2$  solution; control, untreated skin, results are included

Treatment	Zn in SC ( $\mu\text{g}$ )	Zn in receptor solution ( $\mu\text{g}$ )	Zn recovered from donor ( $\mu\text{g}$ ) Post-diffusion	Total Zn recovered		Zn in donor solution (% w/v) Pre-diffusion
				$\mu\text{g}$	% w/v	
Control	$1.0 \pm 0.6$	$1.8 \pm 0.7$	0	$2.8 \pm 0.69$	0.0003	0
5% $\text{ZnCl}_2$	$57 \pm 21$	$2.9 \pm 1.2$	45100	$45160 \pm 4049$	4.5	5.29
50% $\text{ZnCl}_2$	$489 \pm 117$	$2.7 \pm 1.5$	385000	$385492 \pm 60641$	38.5	45.35
Ratio: 50% : 5%	8.58:1	0.93:1	8.54:1	8.54:1	8.56:1	8.57:1

Figure 3 shows zinc penetration into the SC from solutions of 5% and 50% w/v aqueous zinc chloride solutions. In the zinc control experiments, where the skin was treated with de-ionised water that contained non-detectable amounts of zinc, the endogenous levels of zinc found in the stratum corneum were very low. Although it is difficult to view from the

graphs in Fig. 3, the lower stratum corneum layers of skin treated with 5% w/v  $\text{ZnCl}_2$  contained seven-times more zinc than the endogenous levels.

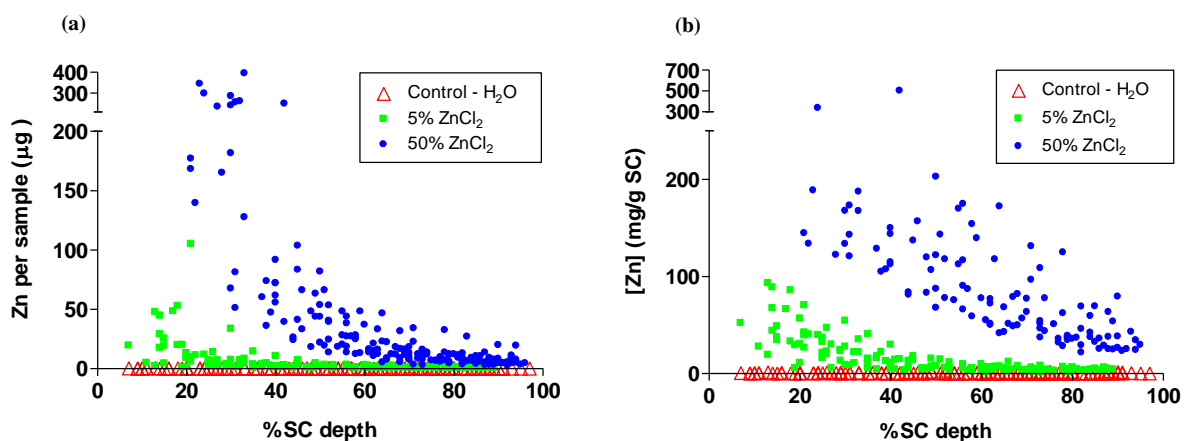


Fig. 3 The uptake of zinc into the stratum corneum expressed as either (a) the amount of zinc per sample, or (b) amount of zinc per unit weight of stratum corneum, as a function of normalised stratum corneum depth. There is a clear distinction observed between the delivery from 5% and, 50% w/v  $\text{ZnCl}_2$  solutions and from the zinc-free controls. Endogenous zinc levels were very low.

Transformation of the amounts of zinc per sample (Fig. 3a) into the quantity of zinc per unit mass of stratum corneum, plotted as a function of % stratum corneum depth, reveals a clear clustering of the treatment groups (Fig. 3b) and that zinc uptake into the stratum corneum from the 50% w/v solution is considerably higher than that from the lower concentration solution. Semi-logarithmic graphs of the same data further delineate the differences perceived (Fig. 4).

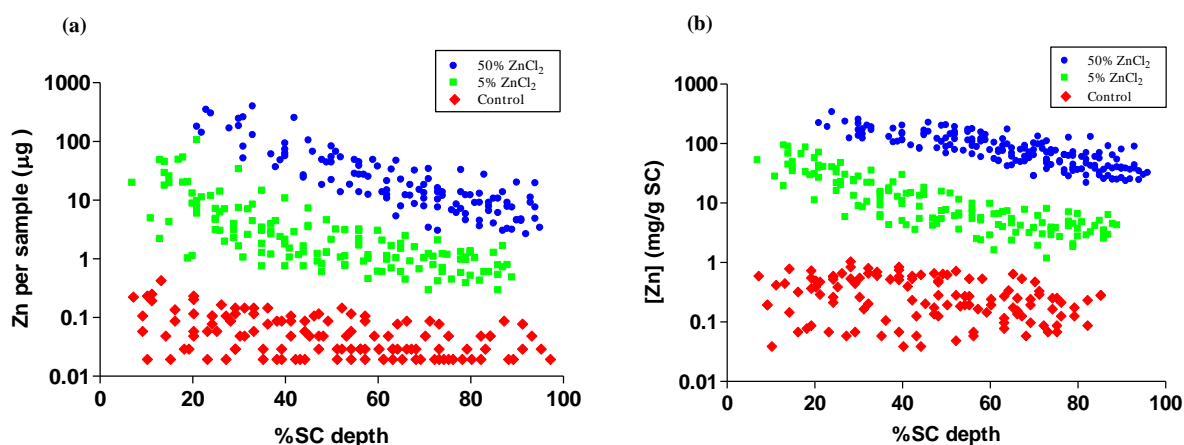


Fig. 4 Penetration profiles of zinc delivered to the SC in vitro expressed in terms of (a) the amount of zinc per tape strip ( $\mu\text{g}$ ), and (b) the quantity of zinc per unit of mass of stratum corneum.

When the results are presented in terms of the cumulative amounts of zinc delivered to the stratum corneum, a similar differentiation between treatments is clear (Fig. 5) The cumulative amounts of zinc recovered in the control stratum corneum and in skin treated with 5% w/v and 50% w/v  $\text{ZnCl}_2$  ranged from 0.24 - 1.9  $\mu\text{g}$ , 33 - 96  $\mu\text{g}$  and 360 - 712  $\mu\text{g}$ , respectively.

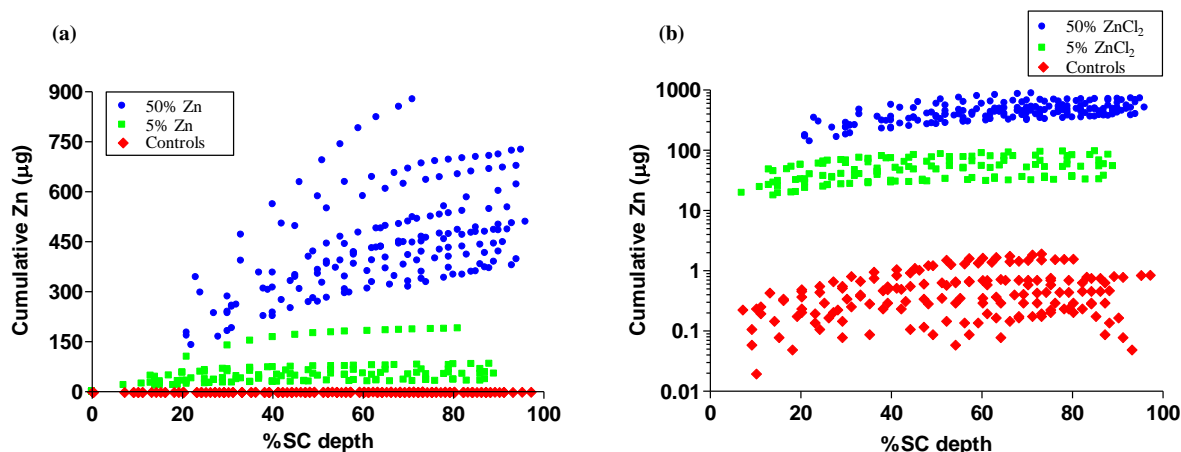


Fig. 5 Cumulative amounts of zinc in the SC as a function of depth into the tissue showing a clear separation between the different treatment groups.

The corresponding mean values ( $\pm$  SD) were  $1.0 \pm 0.6 \mu\text{g}$  ( $n = 10$ ),  $57 \pm 21 \mu\text{g}$  and,  $489 \pm 117 \mu\text{g}$  ( $n = 11$  for each zinc chloride treatment group) (Fig. 6). The absorption of zinc into the SC from a 50% w/v  $\text{ZnCl}_2$  solution was approximately an order of magnitude greater than that from a 5% w/v solution. Overall, uptake represented is approximately 0.04% of the applied amount. The data demonstrate that zinc permeation into the stratum corneum was proportional to  $\text{ZnCl}_2$  concentration and that zinc ions were able to penetrate into the SC albeit with rather poor efficiency. A one-way ANOVA confirmed that there were significant differences between treatment groups with  $P < 0.0001$ .

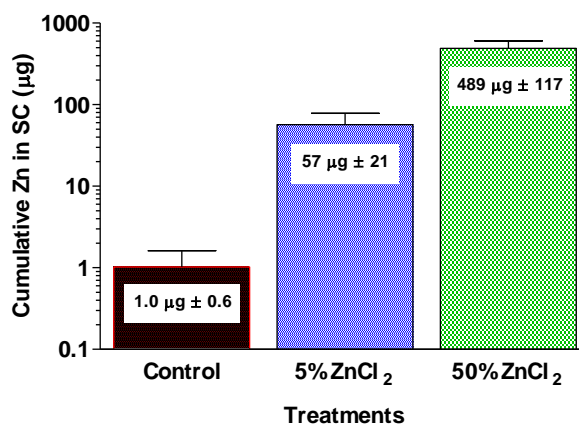


Fig. 6 Mean values ( $\pm$  SD) of cumulative zinc in the SC show significant differences between treatment groups ( $P < 0.0001$ ) ( $n = 10$  for control, and  $n = 11$  for 5% and 50% w/v ZnCl<sub>2</sub> solutions).

The appearance of zinc in the receptor solution of the *in vitro* diffusion cell experiments as a function of time following topical application of either water (control) or 5% and 50% w/v ZnCl<sub>2</sub> solutions is shown in Fig. 7a. Total ‘delivery’ of zinc over 6 hours is in Fig. 7b. The amounts determined (mean  $\pm$  SD) were: control,  $1.8 \pm 0.7$  µg (range 1-2 µg;  $n = 4$ ), 5% w/v ZnCl<sub>2</sub>,  $2.9 \pm 1.2$  µg (range 2-5 µg;  $n = 7$ ); 50% w/v ZnCl<sub>2</sub>  $2.7 \pm 1.5$  µg (range 1-4 µg;  $n = 8$ ). A one-way ANOVA indicated no significant difference between these results ( $P = 0.3551$ ).

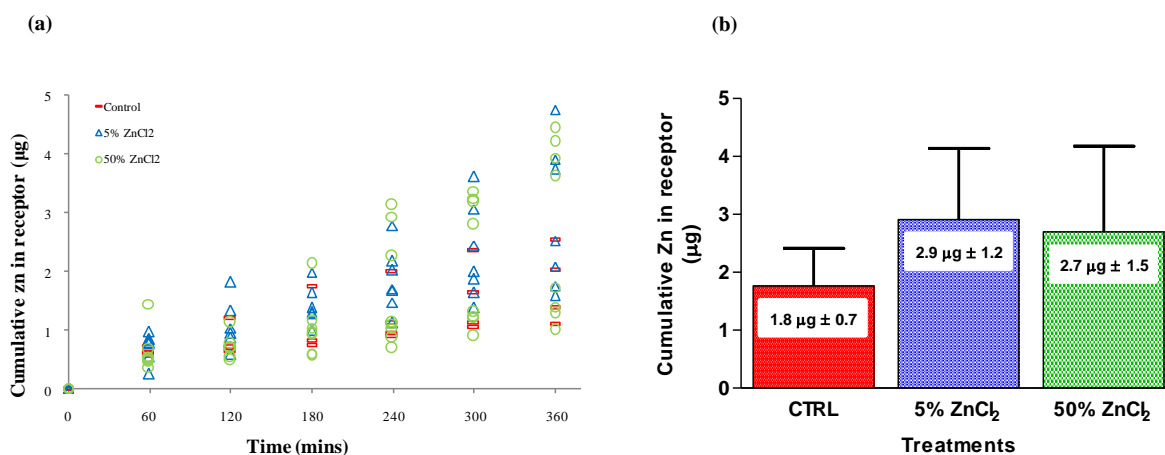


Fig. 7a Hourly sampling of the receptor compartment showed only low levels of zinc. Fig. 7b No statistical significance was observed in zinc content of receptor solutions between treatment groups,  $P = 0.3351$ .

## **Discussion**

The skin uptake experiments performed here using 5% and 50% w/v ZnCl<sub>2</sub> show that zinc ions permeate into the stratum corneum in a manner proportional to the applied concentration: mean cumulative values were  $57 \pm 21 \mu\text{g}$  and  $489 \pm 117 \mu\text{g}$ , respectively, while endogenous zinc in the stratum corneum was only  $1.0 \pm 0.6 \mu\text{g}$ . Despite the significant differences between treatment groups ( $P < 0.0001$ ), the absorption of zinc was low, only approximately 0.04%, reflecting the greater affinity of ZnCl<sub>2</sub> for the aqueous solution (and its high solubility therein) in which it was applied to the lipophilic stratum corneum. Analysis of receptor solutions determined the degree to which zinc penetrated through the barrier. Topical application of 5% and 50% w/v ZnCl<sub>2</sub> solutions resulted in no apparent penetration of zinc ions through the skin. The small quantities of zinc detected almost certainly originated from the skin itself as the levels detected after the zinc treatments overlapped with those from the controls. These results agree with those from an earlier study assessing the absorption of microfine metallic oxides (zinc and titanium) through porcine skin *in vitro*, and which showed that neither element was able to penetrate across the barrier.<sup>(17)</sup> Although *in vitro* experiments can never be interpreted completely unambiguously, porcine skin is a well-recognised model for the human counterpart, and the excision and storage protocol employed here have been shown to have a negligible effect on barrier function.<sup>(27)</sup>

Overall, therefore, the data from this investigation demonstrate that it is possible to deliver zinc into the stratum corneum and that, at least for high concentration ZnCl<sub>2</sub> solutions, the uptake is proportional to concentration. The efficiency of the process, however, is low and the need for a more practical formulation providing improved bioavailability is clear. These conclusions are possible because of the sensitivity and robustness of the atomic absorption assay for zinc in the stratum corneum and donor/receptor samples analysed. The simple calibration of the technique and its specificity are particular advantages of the approach developed.

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## **Chapter Four: Percutaneous Penetration of Zinc from Commercial Formulations**



## **Chapter Four**

### **Percutaneous Penetration of Zinc from Commercial Formulations**

#### **Summary**

*Background* - Many topical zinc preparations are available on the market for the treatment of dermatological conditions. Their purpose, however, is to act as a physical, mechanical barrier that resides on top of the skin to protect it from contact with irritants such as faeces or urine in the case of nappy rash (diaper dermatitis).

*Objectives* - To investigate the *in vitro* delivery of zinc from three commercial formulations; zinc and castor oil cream (7.5% w/w ZnO), Sudocrem<sup>®</sup> (15.25% w/w ZnO) and Morhulin<sup>®</sup> (38% w/w ZnO) currently available on the market

*Methods* - Porcine abdominal skin was subjected to passive diffusion with an excess of formulation for six hours in a Franz diffusion cell. Alternate transepidermal water loss measurements and tape stripping was carried out on the skin followed by the extraction of zinc using acetic acid and quantification by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The control consisted of passive diffusion with deionised water.

*Results* - Of the three commercial formulations tested, the lowest and highest uptake of zinc into the stratum corneum was from zinc and castor oil cream ( $8 \pm 6 \mu\text{g}$ ) and Morhulin<sup>®</sup> ointment ( $58 \pm 35 \mu\text{g}$ ), respectively. Amounts of zinc measured from the controls were low,  $0.9 \mu\text{g} \pm 0.5$ . One-way ANOVA revealed a significant difference of  $P < 0.0001$  between treatment groups. Amounts of zinc measured in the receptor solutions were low and no significant difference was detected between treatments with  $P = 0.16$ .

*Conclusions* - The uptake of zinc into the stratum corneum from Morhulin<sup>®</sup> ointment was highest. Amounts of zinc delivered to the skin appeared to be dependent on the applied dose of zinc oxide. However, considering the concentrations of zinc contained in the formulations, uptake of zinc into the skin is low. This supports the need for the development of a novel topical formulation to aid reparation of the damaged skin barrier in atopic eczema. Further, no penetration of zinc into the receptor solution indicates a negligible risk of systemic toxicity from the percutaneous absorption of these formulations.

Keywords: Zinc oxide, tape stripping, percutaneous penetration, inductively coupled plasma-atomic emission spectroscopy (ICP-AES) Zinc & castor oil cream, Sudocrem<sup>®</sup>, Morhulin<sup>®</sup>

## **Introduction**

Current treatments for eczema mainly revolve around emollients, topical corticosteroids, topical calcineurin inhibitors, antipruritics and antihistamines.(1, 2) Emollients are prescribed first and foremost in the treatment of eczema to aid rehydration of the xerotic skin barrier.(3, 4) Emollient therapy helps control flare-ups of eczema and is expected to be used long term to keep the skin moist and supple. They should be used at least twice a day and on a more regular basis on exposed areas such as the hands and face.(2-4) However, emollient therapy only serves three purposes; (i) rehydration of the stratum corneum to reduce dryness and therefore, improve flexibility, softness and decrease the development of fissures in the skin, (ii) trapping moisture in the skin and thus reducing water loss by creating a lipid film over the surface, and (iii) provision of limited protection against assault from external irritants.(3)

Topical corticosteroids are also commonly used to control flare-ups in eczema.(1, 2, 4-6) Though they decrease inflammation and relieve pruritus (which reduces the frequency of scratching in patients), long term use is not advised due to well recognised side effects.(2, 6-8). Topical glucocorticoids, for example, betamethasone 17-valerate, prednicarbate and mometasone furoate, have been well known to cause skin atrophy.(8, 9) Other side-effects include acne, striae, ulcerations and even steroid rebound or addiction.(7) Topical calcineurin inhibitors such as cyclosporin, tacrolimus and pimecrolimus control the disease state by suppressing the immune response in eczema.(2, 6, 10) However, adverse effects include, but are not limited to, increased permeability of the skin barrier and suppress T-lymphocyte responses which may lead to increased rates of infection and malignancies.(4, 6, 11, 12) Antipruritics and antihistamines offer sedative effects and also control itching and inflammation which lead to help relieve discomfort caused by irritants and allergens that pass through the skin barrier.(1, 2, 6)

The majority of treatments described here offer relief of symptoms only after the manifestations have already caused considerable suffering and discomfort for both the patient and their families. Their intended use is to control exacerbations of lesions, itch and soreness in affected areas. Emollients are useful in providing additional moisture, flexibility to the skin and also relieving sensations of tightness. However, these treatments are fraught with side-effects and do not address the problem that exists in eczema which is

the defective skin barrier. Minute cracks in the stratum corneum allow the facile influx of irritants and allergens into and through the skin to cause discomfort which lead to scratching and ultimately, the development of eczematous lesions.(5, 13-15) To prevent or decrease episodes of flare-ups in the disease would require a means to repair the skin barrier and not just to treat the symptoms once they have manifested. One method of doing so would be to deliver zinc to the skin barrier to aid reparation and restoration of its integrity. As mentioned briefly in Chapter 2, deficiencies in zinc are manifested as dermatological disorders such as skin lesions (observed in eczema), epidermolysis bullosa simplex and delays in the healing of wounds.(16, 17) Further, research has revealed that zinc accumulates in wounds during healing and that rates of healing in damaged tissues are increased after the administration of zinc supplements.(18, 19) Since evidence support the fact that zinc plays such an essential role in the skin during epithelialisation and maintenance, it would be logical to develop a novel topical formulation for the treatment of the skin barrier in eczema. Considering that there are already many commercial topical zinc preparations available on the market today, and also the fact that zinc oxide (the active ingredients in these creams and ointments) is notoriously insoluble (0.16 mg/100 ml water), it would be pertinent to test the uptake of zinc from these products and eventually compare their performance with that of novel formulations prepared during the course of this thesis.(20)

For this study, three topical zinc preparations were purchased; zinc and castor oil (Thornton and Ross Ltd.), Sudocrem<sup>®</sup> (Forest Tosara Ltd.) and Morhulin<sup>®</sup> ointment (Actavis UK Ltd.) and subjected to passive diffusion *in vitro* on porcine abdominal skin followed by tape stripping. The zinc content of each tape strip was quantified by inductively coupled plasma-atopic emission spectroscopy.

## **Materials and Methods**

### **Materials**

Chemicals: Sodium chloride, disodium hydrogen phosphate, potassium phosphate, and potassium chloride were purchased from Acros Organics (Belgium). Zinc and Castor oil cream BP (zinc oxide 7.5% w/w, virgin castor oil 50.0% w/w, cetostearyl alcohol, white beeswax (E901), arachis oil) was purchased from Thornton & Ross Ltd. (Huddersfield, UK), Morhulin<sup>®</sup> Ointment (zinc oxide BP 38% w/w, cod-liver oil BP 11.4% w/w, liquid paraffin BP, yellow soft paraffin BP, lanolin anhydrous BP, castor oil BP, diluted sodium hypochlorite solution) from Actavis UK Ltd (Devon, England, UK) and Sudocrem<sup>®</sup> (zinc oxide Ph.Eur. 15.25%, benzyl alcohol BP 0.39%, benzyl benzoate BP 1.01%, benzyl cinnamate 0.15%, lanolin (hypoallergenic) 4% w/w, purified water, liquid paraffin, paraffin wax, beeswax, microcrystalline wax, sodium benzoate, linalyl acetate, propylene glycol, citric acid, butylated hydroxyanisole, sorbitan sequeioleate, lavender fragrance) from Forest Tosara Ltd. (Dublin).

### **Skin preparation**

Porcine skin obtained from a local piggery (B & J Pigs Ltd., Somerset, UK) was cleaned under cold running water and dermatomed to a thickness of approximately 750  $\mu\text{m}$  (Zimmer<sup>™</sup> Electric Dermatome, Dover, OH, USA). The pieces of skin were then wrapped in Parafilm<sup>™</sup>, placed into a sealed bag and stored in a freezer at -20°C until required.

### **Experimental Procedure**

Frozen porcine abdominal skin was thawed and visually inspected for defects and punctures. Excess hair was carefully trimmed and the skin was rinsed under de-ionised water then mounted into vertical Franz diffusion cells (PermeGear, Inc., Bethlehem, PA, USA) with the external SC surface facing the donor chamber. The area available for transport was approximately 3  $\text{cm}^2$ . The receptor chambers (volume ~ 6 ml) were filled with phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl) at pH 7.4. To verify skin integrity and allow thermal equilibration, the cells were inverted and observed for leaks. After 30 minutes the cells were returned to the upright position and connected to a water bath set at 32 °C and a syringe pump (“Genie” Kent programmable syringe pump, Kent Scientific Corporation, Litchfield, CT, USA). Thermal equilibration took place whilst the formulation was being applied and initiation of the experiment began



after application of the formulation (using a gloved cotton bud) was complete and the donor compartment covered with Parafilm<sup>TM</sup>. The receptor solution was stirred magnetically. 1 ml samples were collected and replaced with fresh buffer at 60 minute intervals over six hours. Following passive diffusion, the skin was removed from the cell and the cream was gently wiped off using a dry paper towel and three isopropyl alcohol wipes and stored between Parafilm<sup>TM</sup> sheets until the next procedure. For tape stripping, the skin was pinned to a polystyrene board and a circular template was attached to demarcate the boundary. Baseline transepidermal water loss (TEWL) was measured (Biox Aquaflux AF102, Biox Systems Ltd., London, UK) and the skin was subjected to multidirectional sequential tape stripping with adhesive tapes (Scotch No. 845 book tape, 3M, St. Paul, MN, USA) that had been previously weighed using a 0.1 µg precision balance (Sartorius AG SE-2F semi-microbalance, Sartorius AG, Göttingen, Germany) and electrically discharged (anti-static device, Eltex, Germany). TEWL measurements were made after every other tape strip and monitored as an indicator of total SC removal (when TEWL ~ 100 gm<sup>-2</sup>h<sup>-1</sup>). The procedure was carried out using zinc and castor oil cream (7.5 % ZnO), Sudocrem<sup>®</sup> (15.25% ZnO) and Morhulin<sup>®</sup> (38% ZnO). Mean doses applied were 0.49 ± 0.04 g. For the controls, deionised water was used in place of the cream.

### Sample Analysis by Inductively Coupled Plasma-Atomic Emission Spectroscopy

After tape-stripping, the tapes were re-weighed to allow the mass and thickness of SC removed to be calculated. The zinc was extracted from the tapes by shaking overnight (IKA HS 260 Basic shaker, IKA<sup>®</sup> Werke GmbH & Co., KG, Germany) in 10% acetic acid. Prior to analysis, all samples were appropriately diluted with 10% acetic acid to facilitate analysis using an inductively coupled plasma sequential atomic emission spectrometer (ICP- AES) (JY Horiba Ultima, Japan) fitted with a Burgener T2100 Nebulizer and JY Horiba AS421 autosampler set at a wavelength of 213.9 nm. Calibration standards were prepared from dilution of 1000 ppm Primar F Zinc Standard from Fisher Scientific Ltd. UK (Loughborough, Leicestershire, UK). All solutions were prepared with deionised water, 18.2 MΩ-cm (NANOpure<sup>®</sup> DIamond<sup>TM</sup> Life Science (UV/UF) Ultrapure Water System, Barnstead International Dubuque, Iowa, USA).

### Data Analysis and Statistics

SC thicknesses were calculated from the TEWL measurements recorded during tape stripping using a modified form of Fick's 1<sup>st</sup> Law of diffusion as described previously. Normality tests, non-linear regression and statistical analyses were performed using GraphPad Prism<sup>®</sup> 4.00 software (Graph Pad Software Inc., San Diego, CA, USA). Statistical significance between multiple data sets was determined via one-way ANOVA. The confidence interval was adjusted to 95% and the significance level was set at  $P < 0.05$ . Outliers, determined by Grubb's test (<http://www.graphpad.com/quickcalcs/index.cfm>), were removed from data analysis.

### Results

The penetration profiles of zinc delivered from the three commercial preparations as a function of normalised stratum corneum depth are shown in Fig.1. Delivery of zinc from the Morhulin<sup>®</sup> ointment (38% w/w ZnO) is clearly the highest (blue). The zinc and castor oil cream (7.5% w/w ZnO) (green) performed less well and overlaps with the controls (open circles). No clear segregation between treatment groups was observed.

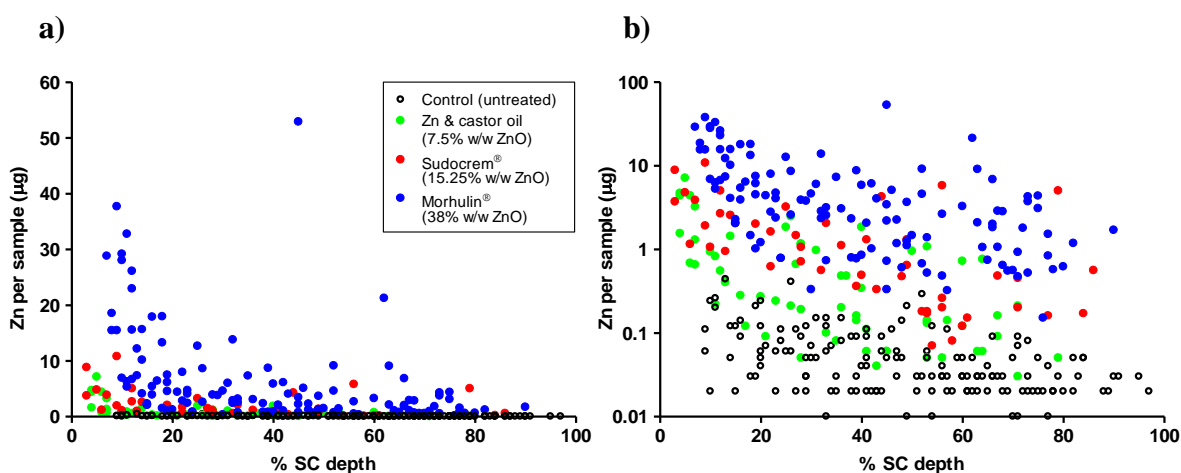


Fig. 1 The amounts of zinc per tape strip is expressed as a function of normalised stratum corneum depth showing endogenous zinc from the controls (untreated) to be lowest and that from Morhulin<sup>®</sup> the highest.

Fig. 2 shows the transformation of the amounts of zinc per sample into the quantity per unit mass of stratum corneum plotted as a function of normalised depth

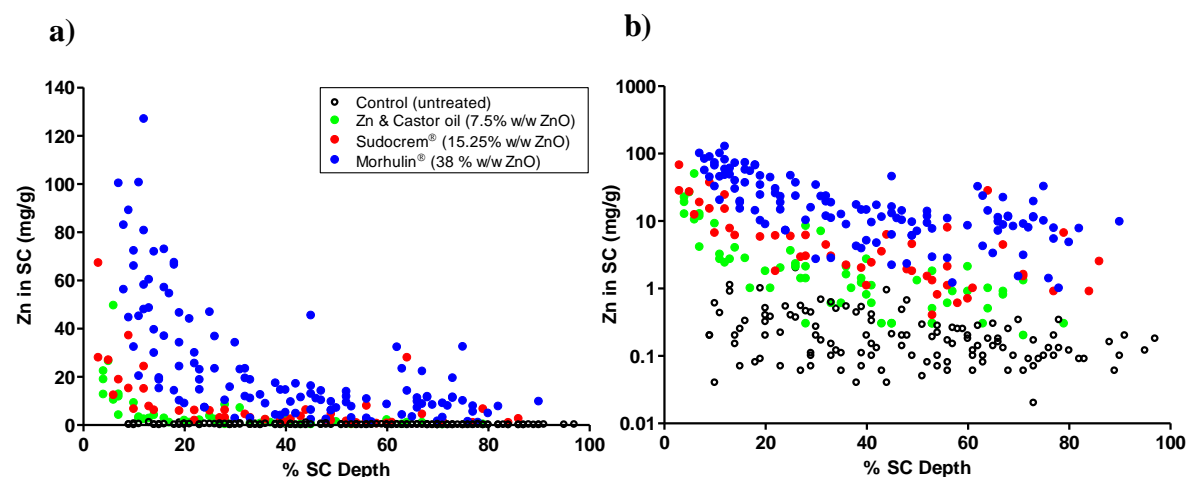


Fig. 2 The uptake of zinc into the stratum corneum expressed as amount of zinc per unit weight of stratum corneum, as a function of normalised stratum corneum depth. Zinc extracted from the controls (untreated) and Morhulin® is clearly seen as the lowest and highest amounts, respectively.

The graphs clearly show the Morhulin® (blue) and control data (untreated) (open circles) begin to separate from other treatments (Fig. 2) with the former and latter being the lowest and highest amounts of zinc contained in the stratum corneum, respectively.

Expressing the cumulative amounts of zinc delivered into the stratum corneum as a function of stratum corneum depth is shown in Fig. 3. The control group (open circles) is almost entirely separate from the treated data (Fig. 3b). This is also true of the Morhulin® data (blue). Zinc and castor oil cream (green) and Sudocrem® (red) data are obviously overlapped with no differentiation into their respectively treatment groups. The highest amount of zinc extracted from skin treated with Morhulin® was approximately 100-times of that from endogenous sources.

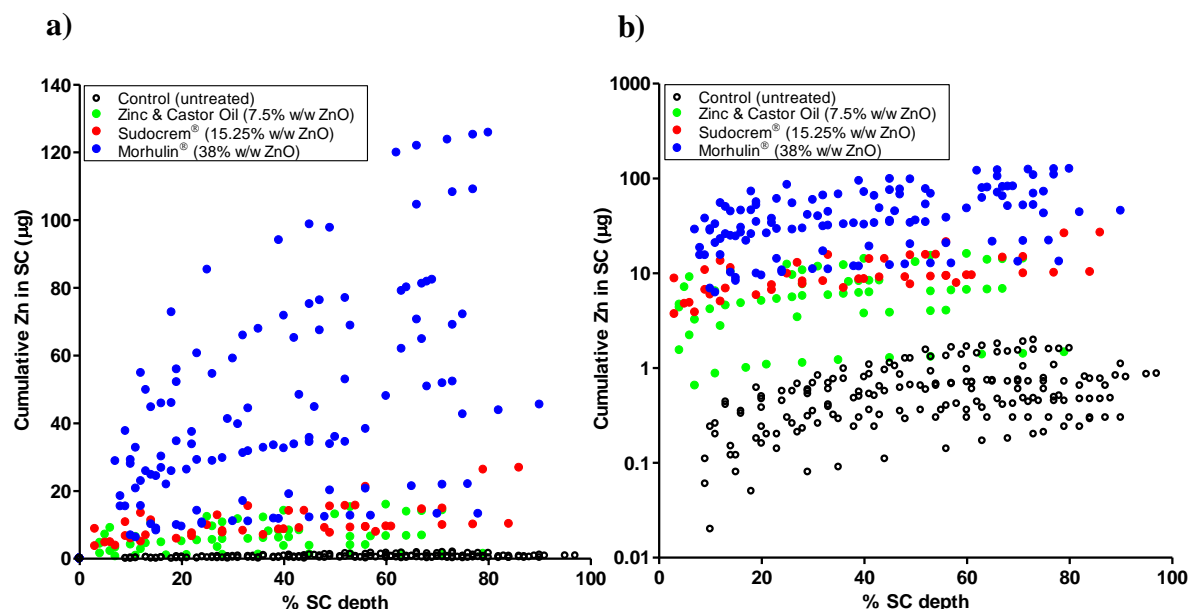


Fig. 3 Cumulative amounts of zinc in the stratum corneum as a function of depth into the tissue showing zinc extracted from the control and Morhulin® treated skin to be almost entirely separated from the other treatment groups.

When represented as a bar graph, cumulative amounts of zinc in the stratum corneum (mean  $\pm$  SD) can be seen to increase with the applied dose of formulation applied, i.e., zinc uptake into the stratum corneum increased with the applied dose (Fig. 4). Statistical analyses using one-way ANOVA detected a significant difference between treatment groups ( $P < 0.0001$ ).

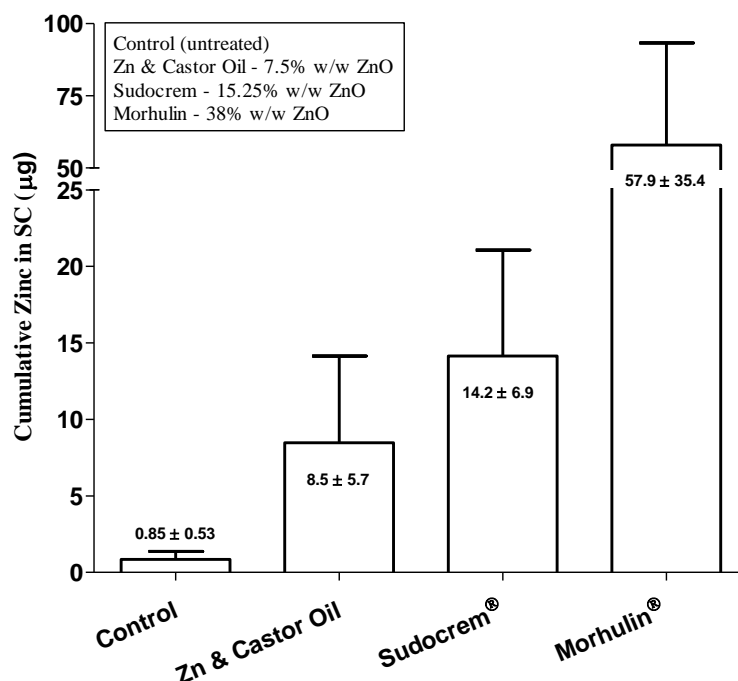


Fig. 4 Cumulative amounts of zinc extracted from the stratum corneum following six hours passive diffusion with commercial formulations. The control was carried out using deionised water. The amounts of zinc extracted from the stratum corneum appeared to increase with concentration applied. One-way ANOVA revealed a significant difference between treatment groups ( $P < 0.0001$ ) ( $n = 11$  for control,  $n = 6$  for Zinc & castor oil cream and Sudocrem® and  $n = 13$  for Morhulin®).

For the receptor solutions, overlap between treatment groups was observed (Fig. 5). Amounts of zinc detected for the control, Zinc and castor oil cream, Sudocrem® and Morhulin® were  $3.51 \pm 1.02 \mu\text{g}$ ,  $4.13 \pm 0.59 \mu\text{g}$ ,  $4.49 \pm 1.13 \mu\text{g}$  and  $4.29 \pm 0.80 \mu\text{g}$ , respectively. One-way ANOVA did not reveal a significant difference between treatment groups ( $P = 0.16$ ).

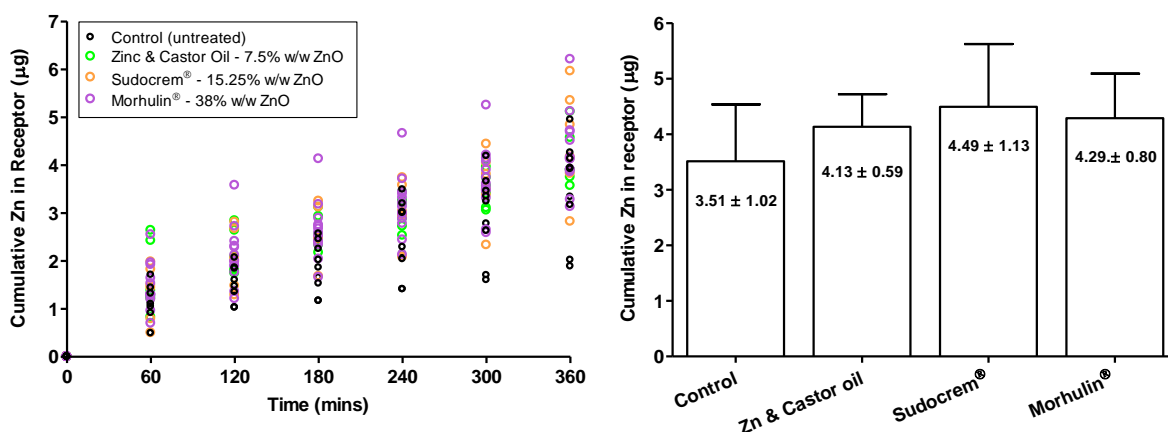


Fig. 5 Samples taken every hour from the receptor solution revealed only low amounts of zinc. No significant difference was observed in zinc content receptor solutions between treatment groups ( $P = 0.16$ ).

## Discussion

This study was carried out to investigate the uptake of zinc into the stratum corneum from commercially available formulations, zinc and castor oil (7.5% ZnO), Sudocrem® (15.25 % ZnO) and Morhulin® (38% ZnO) and to compare these results with those from novel formulations described in Chapter 5.

The results clearly show that zinc uptake from Morhulin® was the highest followed by Sudocrem® and zinc and castor oil where the amounts of zinc extracted from the stratum corneum were  $57.9 \pm 35.4 \mu\text{g}$ ,  $14.15 \pm 6.92 \mu\text{g}$  and  $8.47 \pm 5.67 \mu\text{g}$ , respectively. Only  $0.85 \pm 0.53 \mu\text{g}$  was extracted from the control. No clear segregation between treatment groups was observed (Fig.3). Despite the overlapping of treatments, a significant difference was detected between groups ( $P < 0.0001$ ). The uptake of zinc into the stratum corneum from zinc and castor oil cream and Sudocrem® appeared to be proportional to zinc oxide concentration. Although zinc uptake from Morhulin® ointment was the most efficient, it should be noted that the concentration of zinc oxide in the formulation was highest at 38% w/w. However, while the concentration of zinc oxide in Morhulin® ointment is approximately 2.5 times that of Sudocrem®, but the difference in uptake between the formulations was four-fold. The amount zinc penetrated into the stratum corneum can be misleading because only when the data is represented in terms of percentage of the applied dose (i.e., normalisation) can comparisons in efficiency be made between formulations. The uptake of zinc into the stratum corneum presented as such for zinc and castor oil,

Sudocrem<sup>®</sup> and Morhulin<sup>®</sup> was 0.02%, 0.02% and 0.03%, respectively. The penetration of 5% and 50% w/v zinc chloride in Chapter 3 was approximately 0.04%.

When comparing absolute amounts of zinc penetrated into the stratum corneum from the creams with that of the zinc chloride solutions of approximately the same zinc concentration, it is apparent that latter delivers considerably more zinc than the former. A plausible explanation for this difference can be attributed to the immense difference in solubilities between the zinc oxide (0.14 mg/100 ml) contained in the semi-solid preparations and the aqueous zinc chloride solution (432 g/100 ml). (20, 21)

For the receptor solutions, zinc contents of the formulations were similar to that of the controls. Overlapping of treatment groups was observed (Fig. 5) and no significant difference was detected between treatments ( $P = 0.16$ ).

In conclusion, penetration of zinc from commercial formulations was similar from different preparations and not unlike levels measured from zinc chloride solutions in Chapter 3.

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**Chapter Five: Percutaneous Penetration of Zinc from  
Novel Formulations**



## **Chapter Five**

### **Percutaneous Penetration of Zinc from Novel Formulations**

#### **Summary**

*Background* - The management and treatment for eczema routinely consists of a moisturising regime, the application of corticosteroids, anti-histamine, and if needed, in severe cases, immunosuppressive therapy and oral antibiotics. However, these drugs only treat the symptoms of eczema but not the underlying cause of the flare-ups: the compromised skin barrier. Therefore, the development of a novel topically applied formulation is in progress to aid reparation of the stratum corneum.

*Objectives* - To assess the delivery of zinc from two novel formulations *in vitro*, in porcine abdominal skin.

*Method* - Porcine skin was treated with either a 1% w/w zinc lactate cream or a 2% w/v zinc lactate formulation for six hours in a flow-through Franz diffusion cell. Following passive diffusion, the skin was tape stripped and the zinc was extracted from these samples using 10% acetic acid. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used to quantify the amount of zinc extracted from each tape strip.

*Results* - The uptake of zinc into the stratum corneum from the 1% w/w zinc lactate test cream and from the 2% w/v zinc lactate formulation was  $4.99 \mu\text{g} \pm 1.59$  and  $2.58 \mu\text{g} \pm 1.93$ , respectively. Untreated SC contained only  $0.83 \mu\text{g} \pm 0.59$  of endogenous zinc. A one-way ANOVA revealed a significant difference between these values ( $P = 0.0001$ ). The amounts of zinc detected in the receptor solutions of the diffusion cells following treatment with 1% w/w zinc lactate cream, 2% w/v zinc lactate formulation and deionised water were  $1.74 \mu\text{g} \pm 0.6$  and  $1.52 \mu\text{g} \pm 0.5$  and  $1.39 \mu\text{g} \pm 0.3$ , respectively. No significant difference was found between these results ( $P = 0.43$ ).

*Conclusions* - Uptake of zinc into the stratum corneum from 1% w/w zinc lactate test cream was approximately twice that from the 2% w/v zinc lactate formulation. No zinc penetration greater than background into the receptor solutions suggests that the novel formulations pose little toxicity risk

Keywords: Eczema, topical formulation, zinc lactate, tape-stripping, atomic absorption spectroscopy, passive diffusion.

### **Introduction**

The skin barrier in dermatological diseases such as eczema and psoriasis is compromised due to genetic and environmental factors.(1-4) The genetic aspect dictates the condition of the skin barrier and the predisposition to the development of eczema. Genetic mutations linked to the disease cause an increase in protease activity and a decreased production of intercellular lipid lamellae.(1, 5) These two factors contribute to the premature and facile shedding of corneocytes from the skin causing the barrier to breakdown. In normal, healthy skin, proteases and protease inhibitors work in collaboration in the regeneration and reparation of the skin in homeostasis. In the stratum corneum of healthy skin, low levels of skin proteases cause desquamation of corneocytes at the outer surface. The process is controlled by protease inhibitors that help maintain skin thickness and ensure that only the outer layers are shed.(1) However, in eczema, upregulation of proteases causes premature desquamation to occur and the generation of replacement corneocytes cannot keep up to maintain a constant thickness of the skin barrier. As a consequence the stratum corneum is thinner and more fragile than that of healthy individuals. Intercellular lipid lamellae have two major roles in the structure of the stratum corneum; they act to hold corneocytes together and are responsible for keeping the skin barrier intact and impermeable to the penetration of external irritating agents.(6) In eczema, the production of lipid lamellae is disrupted, undermining the integrity of the stratum corneum and facilitating thereby the influx of irritants and allergen into and through the skin via cracks and fissures.(7) The result is hypersensitivity to substances that are normally innocuous to healthy individuals. Irritation causes the patient to scratch the affected areas leading to the formation of eczematous lesions. Disruption in the generation of intercellular lipid is also related to the production of natural moisturising factor (NMF) that keeps the skin soft and flexible and helps maintain skin barrier integrity.(8) NMF holds water in the stratum corneum, and helps to maintain tight junctions between corneocytes.(9) When the intercellular lipid lamellae are disorganised, NMF is reduced and the skin is xerotic and more prone to developing fissures.

While genetic mutations predispose an individual to eczema, environmental factors increase the likelihood of symptoms being manifest; for example, low humidity, central heating and exposure to a large number of chemicals and foods are known to exacerbate the disease.(10) Exogenous proteases, such as those from *Staphylococcus aureus*

(*S.aureus*) (which is not part of the normal flora of the skin), house-dust mites, and pet dander also contribute to the accelerated breakdown of the skin barrier.(1, 11-14) Other causative factors include washing with hard water, and most importantly, the use of detergents.(1) Sodium lauryl sulphate (SLS) is one of the most commonly used detergents at present and is found in a vast number of personal hygiene products, from toothpaste to shampoo to shower gels and soaps.(15). The use of detergents has been shown to be the most common cause of eczematous flare-ups.(1) In addition, recent research has shown that the application of Aqueous Cream BP, which contains ~1% w/w SLS, on healthy skin *in vivo* decreases skin thickness and increases transepidermal water loss.(16) The natural pH of skin is acidic ('acid mantle').(6) However, increases in skin pH caused by contact with detergents have been reported.(1) Higher skin pH is known to further drive premature desquamation since the activity of skin proteases is optimised in more alkaline conditions. Studies have also shown that when exposed to higher pH, skin barrier function and stratum corneum integrity are affected.(17, 18)

Taking into consideration both genetic and environmental factors that affect the condition of the stratum corneum, it would be logical to treat the impaired barrier to prevent the influx of irritants and allergens instead of treating the symptoms that arise as a result since current treatments for eczema revolve around an emollient regimen and application of various corticosteroid creams (which, when used excessively, cause skin atrophy in addition to other unwanted adverse effects).(19-25)

A new formulation that proposes to aid reparation of the defective skin barrier in eczema has been developed. The novel formulation containing zinc lactate is anticipated to be beneficial in three ways. Firstly, the vehicle, a water in oil emulsion, would help alleviate the dryness experienced in eczema by providing water to rehydrate the skin, and oil to retard further water loss. Secondly, zinc lactate in the formulation should reduce premature desquamation of the stratum corneum by neutralising excess proteases. Significant pieces of research have shown that zinc has the ability to inhibit stratum corneum chymotryptic enzyme (SCCE or kallikrein 7) and stratum corneum tryptic enzyme (SCTE or kallikrein 5), the main proteases that are responsible for premature desquamation when over expressed in eczema.(26, 27) Zinc is also a known beneficial trace element in the treatment of wounds. Research has shown that topical application of zinc was more effective in promoting the healing of wounds than oral zinc supplements (28-30). Previous studies

## **Percutaneous Penetration of Zinc from Novel Formulations**

have also shown that zinc oxide decreased the attachment of *S. aureus* to plastic tissue-culture coverslips.(31) Thirdly, the formulation is approximately pH 4.5. In theory, the acidity should help restore the natural pH of skin (between 4.5 and 6) and decrease skin protease activity which is at an optimum at higher, more alkaline pH as experienced in eczema. Increased protease activity is known to alter skin barrier function, stratum corneum integrity and cause the degradation of lipid processing enzymes.(17) A decrease in skin pH also reduces skin colonisation of *S. aureus* because the production of sphingosine, a natural antimicrobial agent, occurs at low pH. This should also reduce premature desquamation since *S.aureus* is a known secondary protease.(32, 33) Further, it has been reported that when skin pH is below 5, the colonisation of *S. Aureus* is suppressed whereas higher pH promotes growth.(34, 35) Hence, lactic acid in the new formulation should help reduce skin inflammation and infection related to the increased presence of *S. aureus*.(35) Moreover, a lower skin surface pH is correlated to decreased scaling and higher hydration.(35)

Thus, in this chapter, the penetration of zinc from a novel prototype (2% w/v zinc lactate solution) and semi-solid 1% w/w zinc lactate cream formulation will be assessed.

## **Materials and Methods**

### **Materials**

Chemicals: Sodium chloride, disodium hydrogen phosphate, potassium phosphate, and potassium chloride were purchased from Acros Organics (Belgium). Zinc oxide (puriss grade), glycerol (BP, EP, JP, USP) and DL-lactic acid (Ph.Eur, BP and USP) were all purchased from Sigma-Aldrich (Steinheim, Germany),

### **Skin preparation**

Porcine skin obtained from a local piggery (B & J Pigs Ltd., Somerset, UK) was cleaned under cold running water and dermatomed to a thickness of approximately 750 µm (Zimmer<sup>TM</sup> Electric Dermatome, Dover, OH, USA). The pieces of skin were then wrapped in Parafilm<sup>TM</sup>, placed into a sealed bag and stored in a freezer at -20°C until required.

Zinc Lactate Prototype: Zinc oxide and lactic acid (1:2 molar ratio) were dissolved in 20:80% v/v glycerol/water (gly/H<sub>2</sub>O) to create a 2% w/v zinc lactate formulation at pH 4.5

Zinc Lactate Test Formulation: 1% w/w zinc lactate cream from York Pharma Plc.

### Experimental Procedure

Frozen porcine abdominal skin was thawed and visually inspected for defects and punctures. Excess hair was carefully trimmed and the skin was rinsed under deionised water then mounted into vertical Franz diffusion cells (PermeGear, Inc., Bethlehem, PA, USA) with the external SC surface facing the donor chamber. The area available for transport was approximately 3 cm<sup>2</sup>. The receptor chambers (volume ~ 6 ml) were filled with phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl) at pH 7.4. To verify skin integrity and allow thermal equilibration, the cells were inverted and observed for leaks. After 30 minutes the cells were returned to the upright position and connected to a water bath set at 32 °C and a syringe pump (“Genie” Kent programmable syringe pump, Kent Scientific Corporation, Litchfield, CT, USA). Thermal equilibration took place whilst the formulation was being applied and initiation the experiment began after application of the formulation was complete and the donor compartment covered with Parafilm<sup>TM</sup>. The receptor solution was stirred magnetically. 1 ml samples were collected and replaced with fresh buffer at 60 minute intervals over six hours. Following passive diffusion, the skin was removed from the cell and the cream was gently wiped off using a dry paper towel and three isopropyl alcohol wipes and stored between Parafilm<sup>TM</sup> sheets until the next procedure. For tape stripping, the skin was pinned to a polystyrene board and a circular template was attached to demarcate the boundary. Baseline transepidermal water loss (TEWL) was measured (Biox Aquaflux AF102, Biox Systems Ltd., London, UK) and the skin was subjected to multidirectional sequential tape stripping with adhesive tapes (Scotch No. 845 book tape. 3M, St. Paul, MN, USA) that had been previously weighed using a 0.1 µg precision balance (Sartorius AG SE-2F semi-microbalance, Sartorius AG, Göttingen, Germany) and electrically discharged (anti-static device, Eltex, Germany). TEWL measurements were made after every other tape strip and monitored as an indicator of total SC removal (when TEWL ~ 100 gm<sup>-2</sup>h<sup>-1</sup>). For the controls, deionised water was used in place of the cream.



### Sample Analysis

After tape-stripping, the tapes were re-weighed to allow the mass and thickness of SC removed to be calculated. The zinc was extracted from the tapes by shaking overnight (IKA HS 260 Basic shaker, IKA<sup>®</sup> Werke GmbH & Co., KG, Germany) in 10% acetic acid.

Prior to analysis, all samples were appropriately diluted with 10% acetic acid to facilitate analysis by using an inductively coupled plasma sequential atomic emission spectrometer (ICP- AES) (JY Horiba Ultima, Japan) fitted with a Burgener T2100 Nebulizer and JY Horiba AS421 autosampler set at a wavelength of 213.9 nm. Calibration standards were prepared from dilution of 1000 ppm Primar F Zinc Standard from Fisher Scientific Ltd. (Fig. 1)

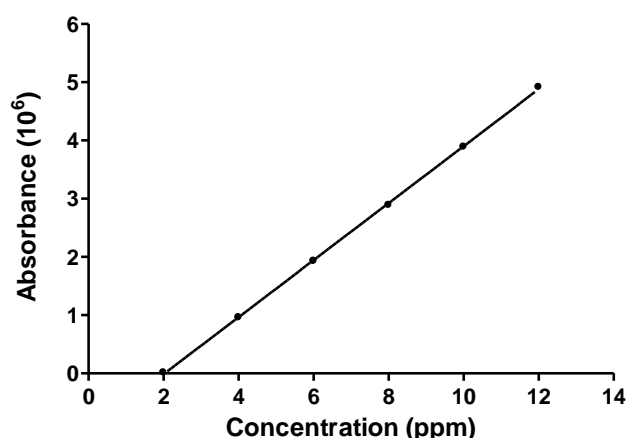


Fig. 1 Calibration curve for zinc standards using ICP-AES (n = 6 per standard). ( $r^2 = 0.9997$ )

To enable an estimation of mass balance, the original donor solutions of 5% w/v and 50% w/v aqueous  $\text{ZnCl}_2$  (not used in passive diffusion experiments) were also diluted and analysed. All solutions were prepared with deionised water, 18.2 M $\Omega$ -cm (NANOpure<sup>®</sup> Diamond<sup>™</sup> Life Science (UV/UF) Ultrapure Water System, Barnstead International Dubuque, Iowa, USA).

### Data Analysis and Statistics

SC thicknesses were calculated from the TEWL measurements recorded during tape stripping using a modified form of Fick's 1<sup>st</sup> Law of diffusion as described previously. Normality tests, non-linear regression and statistical analyses were performed using GraphPad Prism<sup>®</sup> 4.00 software (Graph Pad Software Inc., San Diego, CA, USA). Statistical significance between multiple data sets was determined via one-way ANOVA.

The confidence interval was adjusted to 95% and the significance level was set at  $P < 0.05$ . Outliers, determined by Grubb's test (<http://www.graphpad.com/quickcalcs/index.cfm>), were removed from data analysis.

## Results

The zinc profiles in the stratum corneum of skin treated with 1% w/w zinc lactate cream, 2% w/v zinc lactate and the control (deionised water) are shown in Fig. 2. The amount of zinc extracted from skin treated with the 1% w/w zinc lactate cream (blue) is higher than that from the 2% w/v zinc lactate formulation (red). The control (empty circles) showed the lowest levels of zinc extracted. A semi-logarithmic graph of the same data (Fig. 2b) expands the distribution of the data points but does not show clear differentiation of the treatment groups.

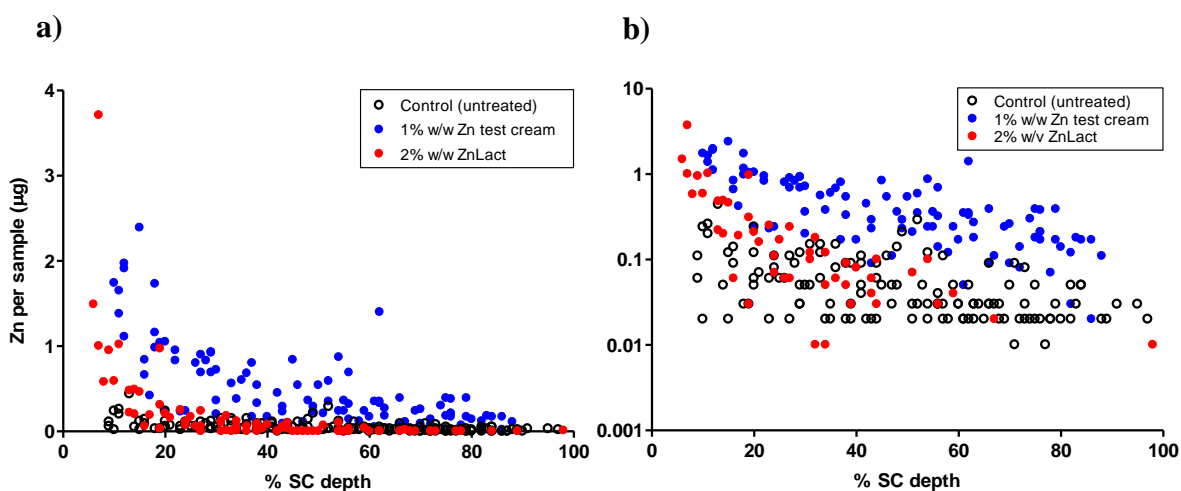


Fig. 2 The uptake of zinc into the stratum corneum expressed as the amount of zinc per sample as a function of normalised stratum corneum depth. Higher levels of zinc were extracted from 1% w/w cream compared to the 2% w/v zinc lactate formulation. Endogenous zinc from the control is seen in the lower regions of the graph (open circles).

The quantities of zinc per unit mass of stratum corneum as a function of stratum corneum depth are shown in Fig. 3. The treatments fall into two groups, which are not clearly separated from each other.

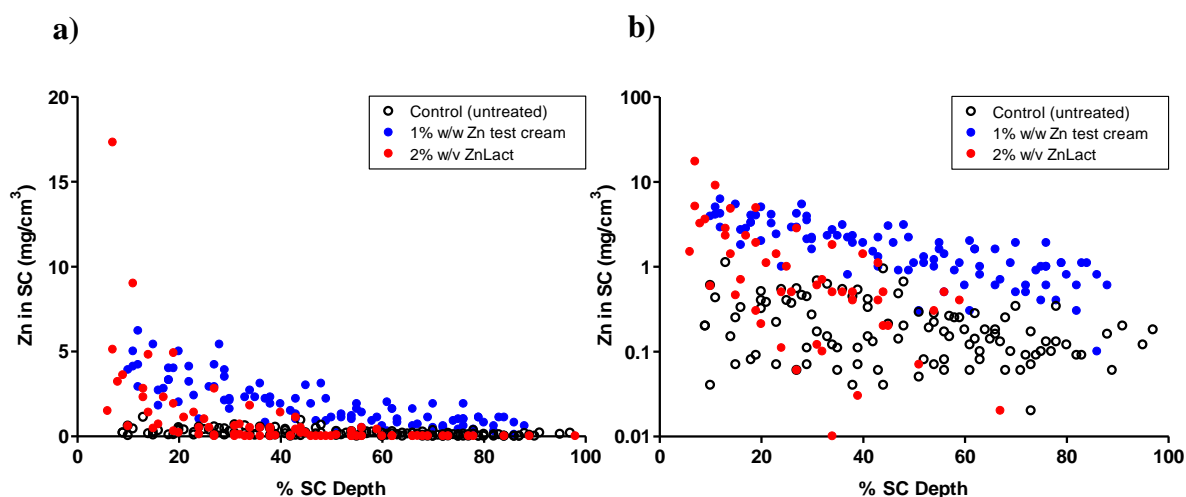


Fig. 3 Penetration profiles of zinc delivered per unit mass of stratum corneum expressed as a function of stratum corneum depth. Separation of the 1% w/w zinc lactate cream data is clearer in the semi-logarithmic plot (b).

The cumulative amounts of zinc in the stratum corneum as a function of stratum corneum depth are shown in Fig. 4. The graphs show that the levels of zinc delivered to the stratum corneum from the two formulations are higher than the control indicating that zinc did penetrate into the skin from the two formulations.

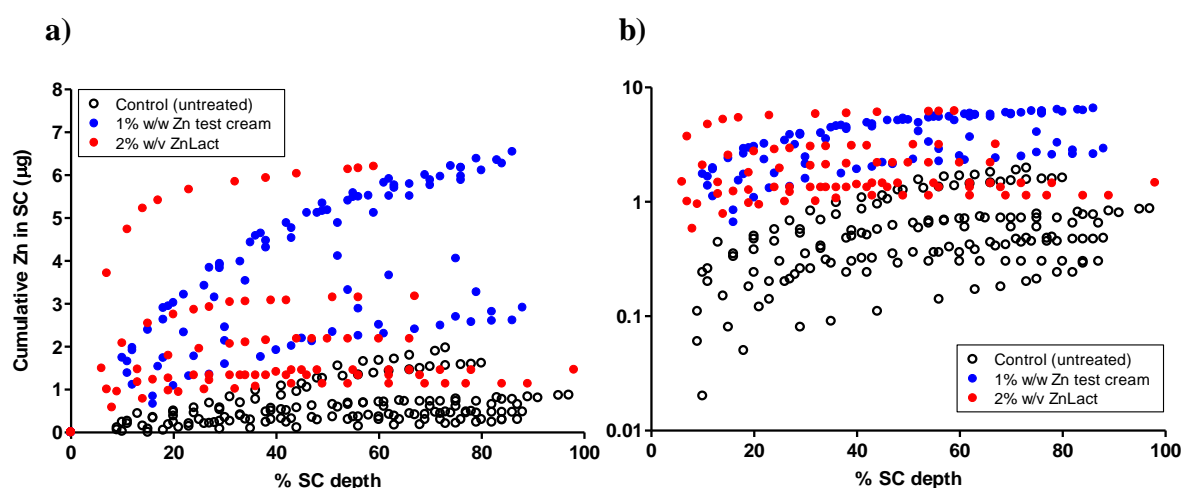


Fig. 4 Cumulative amounts of zinc in the stratum corneum as a function of stratum corneum depth.

The total quantities of zinc recovered in the control, 1% w/w zinc lactate cream and 2% w/v zinc lactate treated skin are shown in Fig. 5 and were  $0.83 \pm 0.59 \mu\text{g}$ ,  $4.99 \pm 1.59 \mu\text{g}$  and  $2.58 \pm 1.93 \mu\text{g}$ , respectively. Zinc delivered from the 1% w/w zinc lactate cream was approximately twice that from the 2% w/v formulation. A one-way ANOVA carried out on the data revealed a significant difference between the treatment groups ( $P = 0.0001$ ).

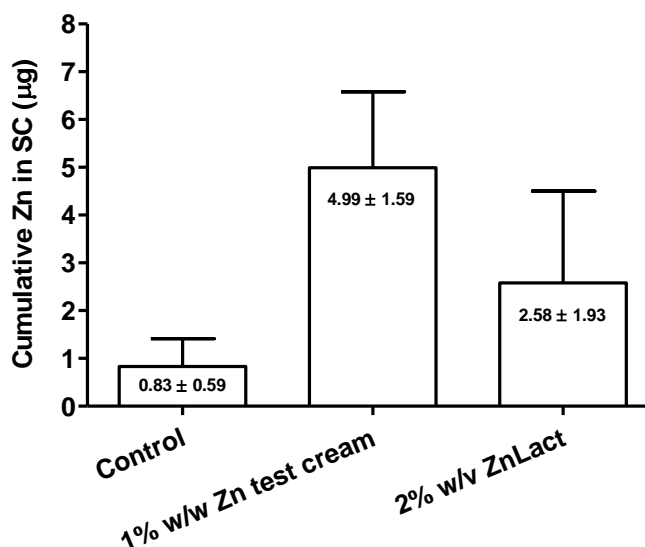


Fig. 5 The cumulative amounts of zinc extracted from untreated skin (control) and skin treated with 1% w/w zinc lactate cream and 2% w/v zinc lactate formulation; the mean ( $\pm$  SD) values are indicated for  $n = 9$ , 10 and 6, respectively.

Tukey's multiple comparison test detected significant differences between control and 1% w/w zinc lactate cream ( $P < 0.001$ ), and also between 1% w/w zinc lactate cream and 2% w/v zinc lactate formulation ( $P < 0.01$ ).

Cumulative amounts of zinc detected in the receptor solutions as a function of time are shown in Fig. 6. Overlapping of the control and treated skin data can be observed (Fig. 6a). Total penetration of zinc for the control and 1% w/w zinc lactate cream and 2% w/v zinc lactate formulation (mean  $\pm$  SD, where  $n = 9$ , 10 and 6) were  $1.74 \pm 0.6 \mu\text{g}$ ,  $1.52 \pm 0.5 \mu\text{g}$  and  $1.39 \pm 0.3 \mu\text{g}$ , respectively. No significant differences were detected between treatment groups using one-way ANOVA ( $P = 0.43$ ).

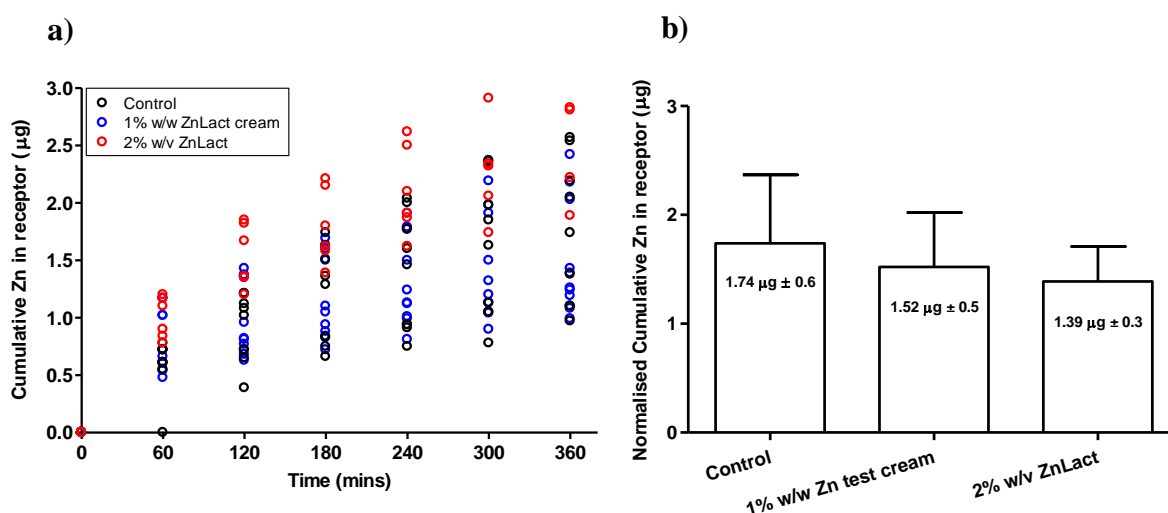


Fig. 6 (a) Hourly samples of receptor compartments showed low levels of zinc. (b) No statistical difference was observed in the zinc content of receptor solutions between treatment groups,  $P = 0.43$ .

## Discussion

The results of many studies have shown that zinc oxide has very limited penetration into the skin. (36-43) In a recent study, ultrafine zinc oxide (Z-COTE) was applied intact human skin *in vitro* and imaged using x-ray spectroscopy and scanning transmission ion microscopy. The results revealed that the penetration of zinc was limited to the outer surface of the skin. Even when the stratum corneum was partially or entirely removed, no penetration of zinc was observed in the deeper skin layers.(44)

In this chapter, the performance of a 2% w/v zinc lactate formulation (the prototype of the resulting cream formulation) and a 1% w/w zinc lactate cream was assessed. The results of this investigation show that the uptake of zinc from a 1% w/w zinc lactate cream (prepared by York Pharma Plc.) into the stratum corneum of porcine abdominal skin was approximately twice that of the 2% w/v zinc lactate formulation, and five-times greater than the endogenous level of the element ( $4.99 \pm 1.59 \mu\text{g}$ ,  $2.58 \pm 1.93 \mu\text{g}$  and  $0.83 \pm 0.59 \mu\text{g}$ , respectively). The amount of zinc penetrated (minus levels detected from endogenous sources) into the stratum corneum as a percentage of the applied dose was approximately 0.07% and 0.004% from the 1% w/w and 2% w/v zinc formulations, respectively. The difference in zinc delivery between the prototype and the cream formulation could be due to differences in compositions of the two formulations. The simple prototype contained

only four components; lactic acid, zinc lactate, glycerol and deionised. However, the cream may have contained penetration enhancers or surfactants that may have facilitated delivery of zinc into the skin. Further, another explanation for the difference in performance could be due to the variation in zinc lactate particle size between formulations. Since this information is not available, it is therefore, not possible to draw firm conclusions based on this hypothesis. Additionally, since the composition of the cream formulation is unknown, these theories can only be speculative.

Amounts of zinc extracted from skin treated with zinc chloride (Chapter 3), as a percentage of the applied dose from both 5% and 50%  $\text{ZnCl}_2$  was 0.04% (where zinc uptake was  $57 \pm 21 \mu\text{g}$  and  $489 \pm 117 \mu\text{g}$ , respectively). The amount of zinc uptake into the skin from the 1% w/w zinc lactate cream was more than twice that value. The amounts of zinc delivered into the skin from these commercial formulations; zinc and castor oil (7.5% w/w  $\text{ZnO}$ ), Sudocrem<sup>®</sup> (15.25% w/w  $\text{ZnO}$ ) and Morhulin<sup>®</sup> (38% w/w  $\text{ZnO}$ ), were  $8.47 \pm 5.67 \mu\text{g}$ ,  $14.15 \pm 6.92 \mu\text{g}$  and  $57.9 \pm 35.4 \mu\text{g}$ , respectively. Expressed as a percentage of the applied dose: 0.02%, 0.02% and 0.03%. So, although Morhulin<sup>®</sup> delivered the highest level of zinc to the skin, when the amounts were normalised against the dose of zinc applied, it is clear that the 1% w/w zinc lactate cream was more effective at delivering zinc to the skin.

In conclusion, the novel 1% w/w zinc cream performed most efficiently and has potential, with further optimisation, to achieve even better delivery. Even though the percent dose delivered was low, all the zinc would have been available in molecular form (the zinc lactate being fully dissolved in the formulation), unlike that taken up from the older preparations. In addition, it would be logical to expect a higher zinc uptake when applied to uninvolved or involved skin of eczema patients.

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**Chapter Six: Delivery of zinc to the skin - Imaging by  
Scanning Electron Microscope**



## **Chapter Six**

### **Delivery of zinc to the skin - Imaging by Scanning Electron Microscope**

#### **Summary**

*Background* - Zinc is widely used in commercial formulations for the treatment of a variety of skin conditions. It is also one of the main ingredients in many sunscreen preparations. In previous chapters of this thesis, percutaneous penetration of zinc from a range of vehicles has been investigated. However, the effects of formulation and surface cleaning the topographical or distribution of zinc on and in the skin has not yet been fully characterised.

*Objectives* - To investigate the effects of different zinc formulations and two surface cleaning procedures on the topographical distribution of zinc on the stratum corneum after passive diffusion.

*Methods* - Porcine abdominal skin was treated with deionised water (control) and several zinc-containing formulations: 5% and 50% w/v aqueous zinc chloride, Morhulin<sup>®</sup> (38% w/v ZnO), and 2% w/v zinc lactate in either 20:80 or 80:20 v/v glycerol/water. Post treatment, the skin surface was cleaned either by dry wiping, rinsing with deionised water, or swabbing with an isopropyl alcohol wipe. All treated skin samples were then imaged using scanning electron microscopy and the presence of zinc was detected using energy dispersive X-ray (EDX) microanalysis. The spatial distribution of zinc was studied by electron mapping of the skin surface.

*Results* - Topographical features observed included areas of skin with either 'rough' or 'smooth' appearance, so-called furrows and hairs/hair follicles. Rinsing skin with treated with zinc chloride solutions removed substantial amounts of the element from rougher skin areas, furrows and hairs (but not from smoother regions of skin). The uptake of larger amounts of zinc from 50% w/v ZnCl<sub>2</sub> than from a 5% w/v solution was clearly shown. Skin treated with Morhulin<sup>®</sup> showed a white film-like deposit on the skin surface. The zinc lactate formulations delivered less zinc into the SC than the ZnCl<sub>2</sub> solutions.

*Conclusions* - EDX spectra and element maps provide useful complimentary information on the uptake and disposition of zinc following its delivery to the skin in different formulations. The presence of zinc was detected on the surface of skin treated with 5 and 50% w/v ZnCl<sub>2</sub>. 50% w/v ZnCl<sub>2</sub> solution delivered more zinc to the skin as shown by EDX spectra and element maps. Rinsing the skin with deionised water proved to remove zinc

more effectively than wiping with a dry paper towel. Of all the formulations tested, 50% w/v ZnCl<sub>2</sub> delivered the highest amount of zinc to the skin.

Keywords: Scanning electron microscopy (SEM), energy dispersive X-ray microanalysis (EDX), electron mapping, zinc chloride, percutaneous penetration

## **Introduction**

It is evident from work previously carried out (and detailed in earlier chapters) that the delivery of zinc to the skin from a variety of formulations penetrates into, and remains largely in the upper layers of the stratum corneum. Since the removal of successive layers of skin by tape stripping only provides the overall amount of zinc delivered into the SC analysed, it is pertinent to investigate whether any localisation of zinc to specific skin areas, or structures occurs. A useful technique for this purpose is electron microscopy yet, while the penetration of zinc oxide (and titanium dioxide) has been the subject of numerous studies (1-6), the application of microscopy to visualise the fate of zinc in the skin has received little attention (7-13). In contrast, most previous studies have primarily focused on the health and safety aspects of applying sunscreens to the skin and have been concerned with whether nanoparticles are able to pass through the epidermis and into the systemic circulation where they may elicit local or systemic toxicity.

In this experimental chapter, scanning electron microscopy and energy dispersive X-ray (EDX) microanalysis have been used to monitor the disposition of zinc on and into the stratum corneum following topical application of various zinc-containing formulations. The imaging undertaken has also allowed any detrimental effects on the skin of the formulations used (e.g., concentrated solutions of zinc chloride) to be assessed.



## **Materials & Method**

### **Materials:**

*Chemicals:* Zinc chloride, 99.995 +% purity was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Morhulin<sup>®</sup> Ointment was from Actavis UK Ltd (Devon, England, UK). Zinc oxide (puriss grade), glycerol (meets BP, EP, JP, USP testing specifications), DL-lactic acid (meets analytical specifications of Ph.Eur, BP and USP) were obtained from Sigma-Aldrich (Steinheim, Germany) Sodium chloride, disodium hydrogen phosphate, potassium phosphate, and potassium chloride were all purchased from Acros Organics (Belgium). Glacial acetic acid (HPLC grade) was from Fisher Scientific.

### **Skin preparation**

Porcine abdominal skin obtained from a local piggery (B & J Pigs Ltd., Somerset, UK) was cleaned under cold running water and dermatomed to a thickness of approximately 750  $\mu\text{m}$  (Zimmer<sup>™</sup> Electric Dermatome, Dover, OH, USA). The pieces of skin were then wrapped in Parafilm<sup>™</sup>, placed into a sealed bag and stored in a freezer at -20°C until required.

Zinc chloride solutions: Aqueous solutions of zinc chloride, 5% w/v and 50% w/v, were freshly prepared for each experiment.

Zinc lactate preparation: Zinc oxide (2% w/v) and lactic acid (0.2% w/v) were dissolved in 20:80% v/v glycerol/water (gly/H<sub>2</sub>O) to create a 2% w/v zinc lactate formulation at pH 4.5. A 80:20% v/v gly/H<sub>2</sub>O zinc lactate formulation was also prepared.

Buffer: Phosphate buffered saline, pH 7.4 was prepared using a standard formula.<sup>1</sup>

All solutions were prepared with deionised water, 18.2 M $\Omega$ -cm (NANOpure<sup>®</sup> DIamond<sup>™</sup> Life Science (UV/UF) Ultrapure Water System, Barnstead International, Dubuque, Iowa, USA).

### **Zinc delivery into SC**

The skin was thawed and visually inspected for defects and punctures. Excess hair was carefully trimmed, the skin was rinsed under deionised water and then cut into three. These tissue samples were mounted between the donor and receptor compartments of three Franz

flow-through diffusion cells (PermeGear, Inc., Bethlehem, PA, USA) with the external SC surface facing the donor compartment. The receptor compartments were filled with PBS at pH 7.4 (volume ~ 6 ml) and the cells were temporarily inverted to verify skin integrity. After approximately 20 minutes the cells were returned to an upright position and the donor solutions (3 ml): (i) deionised water, (ii) 5% ZnCl<sub>2</sub>, and (iii) 50% ZnCl<sub>2</sub> were pipetted into donor chambers. Each donor was covered with Parafilm<sup>TM</sup> and the cells were placed onto a magnetic stirrer and connected to (a) a water bath set at 32 °C and (b) a “Genie” Kent programmable syringe pump (Kent Scientific Corporation, Litchfield, CT, USA). Receptor samples of 1 ml were collected every hour. After six hours of passive diffusion, the donor solutions were discarded and the skin samples dismantled from each cell. Where applicable, the pieces of skin were then cut into two portions one of which was dried using a dry paper towel, while the other was rinsed under de-ionised water. In preparation for inspection under the scanning electron microscope (SEM), the pieces of skin were flash frozen in liquid nitrogen then freeze dried (BOC Edwards Freeze Dryer, NY, USA) overnight. The samples were coated (BOC Edwards Sputter Coater S150B) with a fine layer of gold before analysis under the scanning electron microscope (SEM).

The experiments were repeated using Morhulin<sup>®</sup>, 2% zinc lactate (20:80% gly/H<sub>2</sub>O) and 2% zinc lactate (80:20% gly/H<sub>2</sub>O) as donors formulations all of which were applied in excess.

#### Scanning electron microscopy (SEM)

SEM images were obtained using a JEOL 6480LV SEM (Japan). The microscope was set at a magnification of x350, an accelerating voltage of 20kV, a spot size of 25 and a working distance 10mm. Other parameters were adjusted to optimum values. To collect the X-ray microanalysis data, the microscope was set to an accelerating voltage of 20kV, a spot size of 50 and a working distance 10mm. All other parameters were adjusted to optimum values to give a minimum count rate of 1.3 - 1.5 kcps, and a dead time of < 10%. Spectra were collected for 100 seconds and maps for > 80 frames. The analysis programme used was INCA, produced by Oxford Instruments (UK).

## **Results - Part 1**

### Control - skin treated with deionised water

SEM images of the hair (and hair shaft), skin furrow, smooth and rough areas of skin treated with deionised water are in Fig. 1a-d.

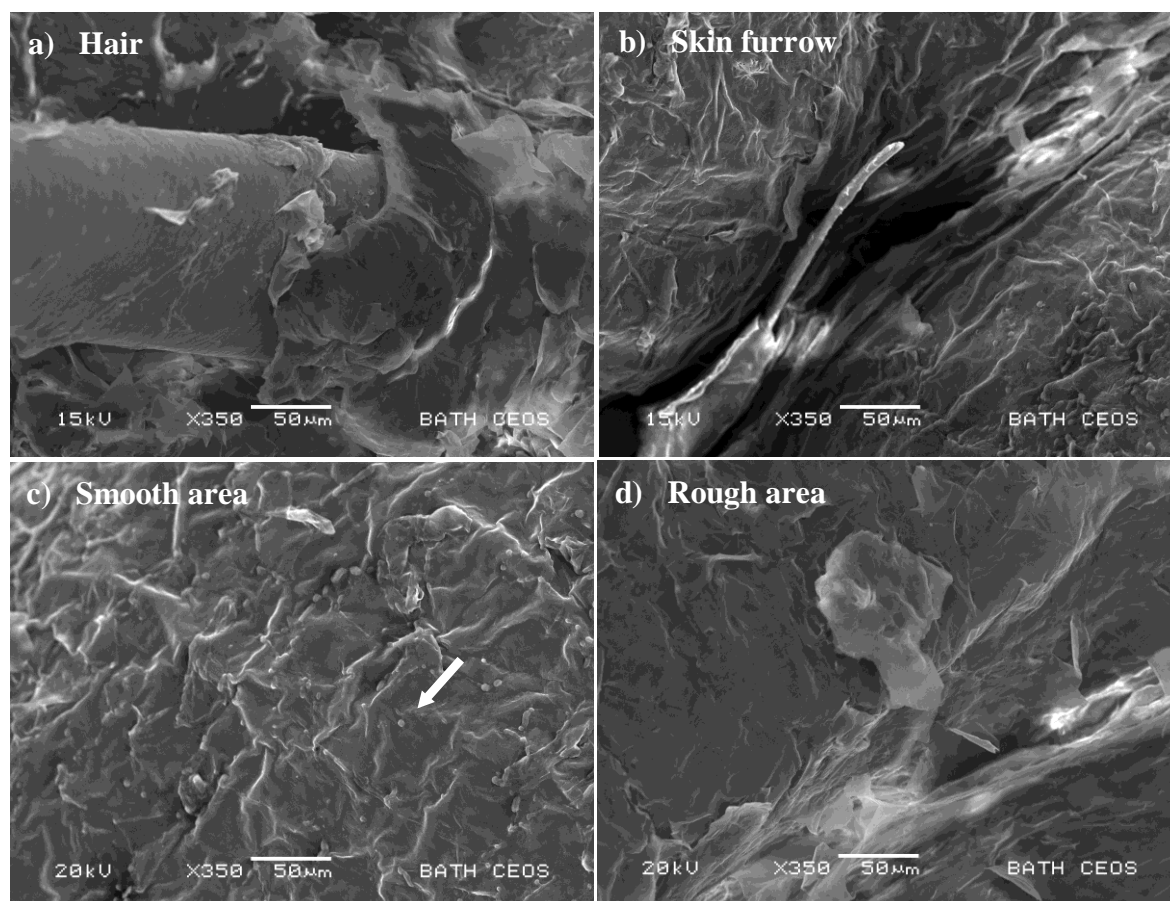


Fig. 1 SEM images of; a) hair, b) skin furrow, and c) smooth and, d) rough areas of untreated skin. The arrow points to an example of an unidentified organic structure present on the skin surface.

Energy dispersive X-ray (EDX) microanalysis revealed elemental profiles on the skin surface. No zinc was detected at any untreated skin sites (Fig. 2).

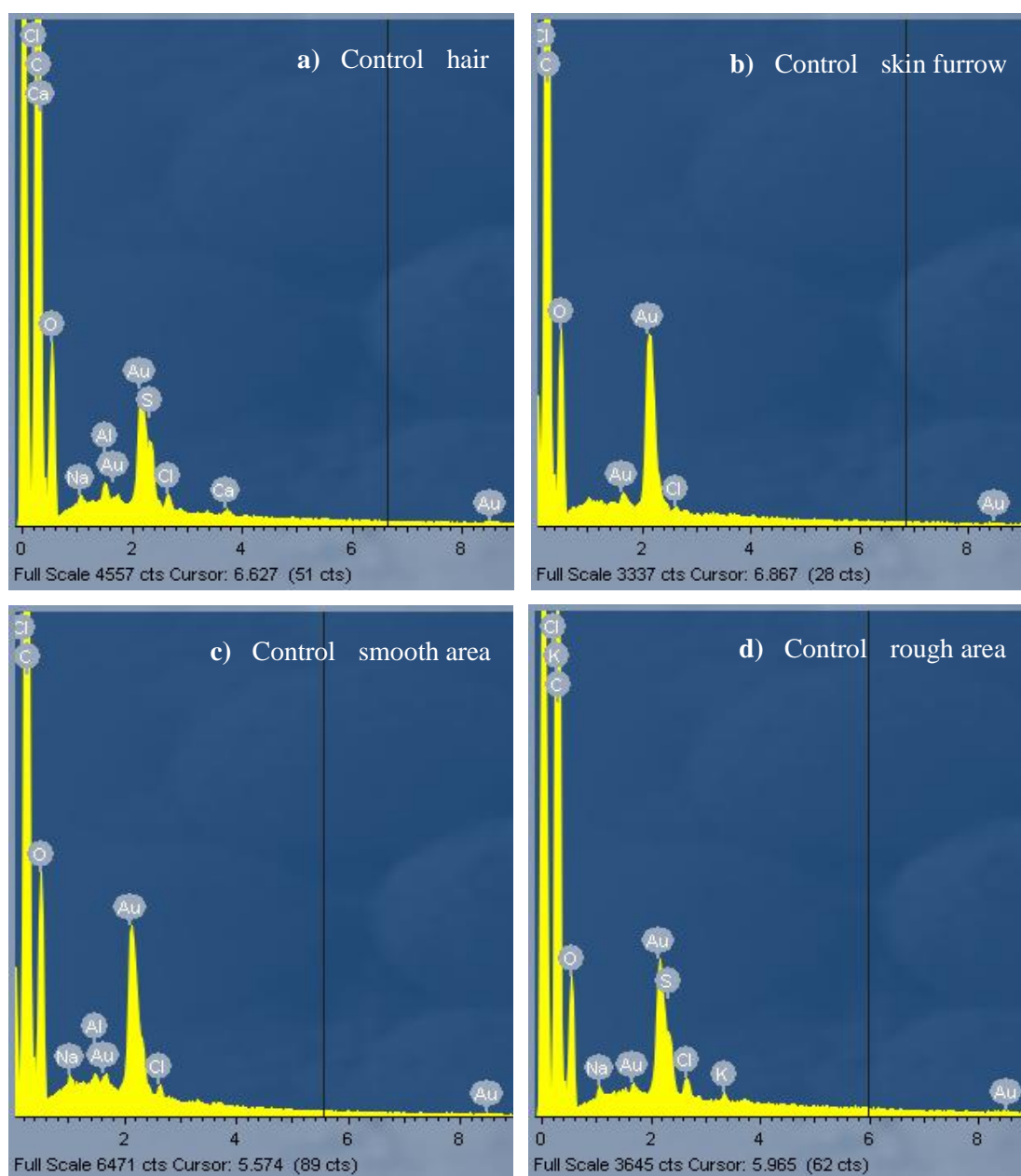


Fig 2. Energy dispersive X-ray microanalysis of a) hair, b) skin furrow, and c) smooth, and d) rough areas of control (untreated) skin. No trace of zinc was detected anywhere.

## **Results - Part 2**

### Disposition of zinc following ZnCl<sub>2</sub> application and surface cleaning

The delivery of zinc to the skin from 5% and 50% w/v ZnCl<sub>2</sub> solutions was investigated. The use of 2 cleaning procedures was also studied: rinsing with deionised water or wiping with a dry paper towel.

### Hair and skin furrows

Zinc deposits were observed on hairs present on skin which had been wiped with a dry paper towel after treatment with 5 and 50 % w/v ZnCl<sub>2</sub> (Fig. 3a-b). Treated skin rinsed with deionised water did not show zinc coating on the hairs and surrounding areas which appeared clean and smooth (Fig. 3c-d). With regard to furrows, no visible change to the topography of the skin was apparent between different zinc treatments after removing the excess with a paper towel (Fig. 3e-f). No significant differences were observed between skin furrows treated with 5% and 50% ZnCl<sub>2</sub> after both cleaning procedures (Fig. 3e-h).

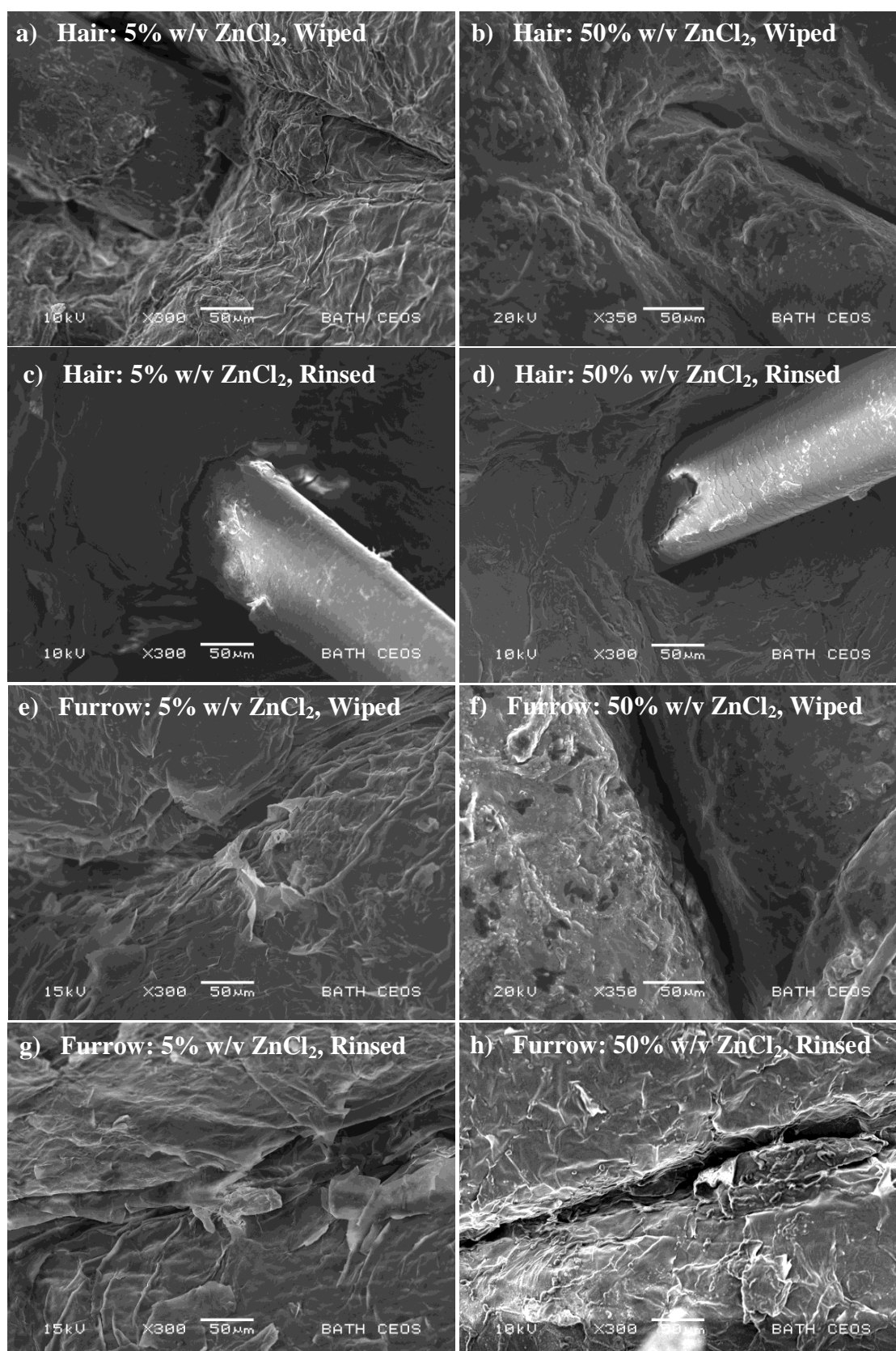
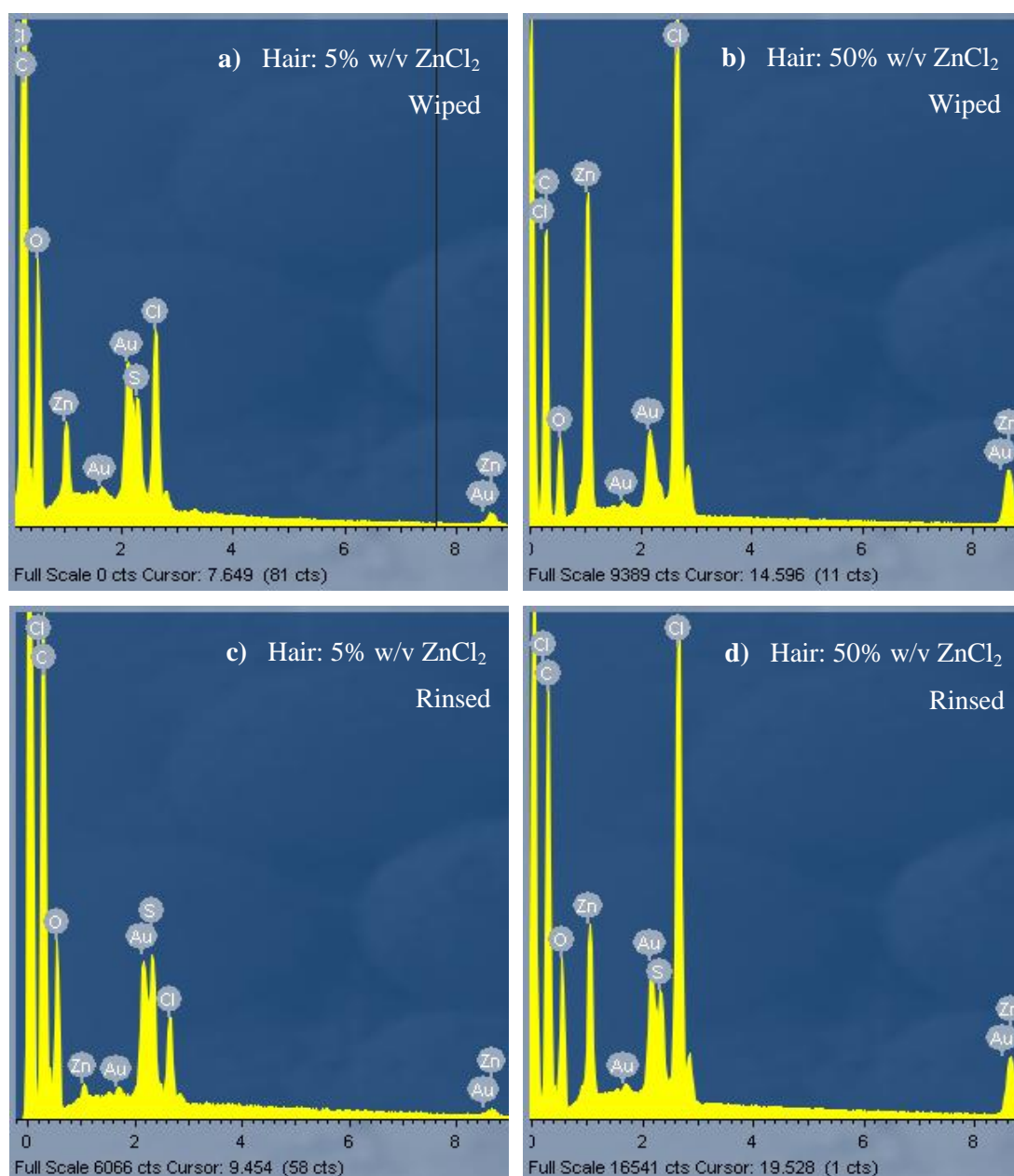


Fig. 3 SEM Images of hair and skin furrows. Left and right panels were treated with 5 and 50 % w/v  $\text{ZnCl}_2$ , respectively. Samples, a), b), e), and f), were wiped dry directly after treatment, while samples c), d), g) and h) were rinsed with deionised water before drying.

Energy dispersive X-ray (EDX) microanalysis spectra showed that levels of zinc delivered to the skin correlated with the concentration applied; i.e., more zinc was deposited/delivered to the skin from the more concentrated solution (Fig.4). Skin samples wiped with a paper towel directly after treatment (Fig. 4a, b, e, f) showed higher levels of zinc (and chlorine) than those rinsed with deionised water (Fig. 4c, d, g, h).



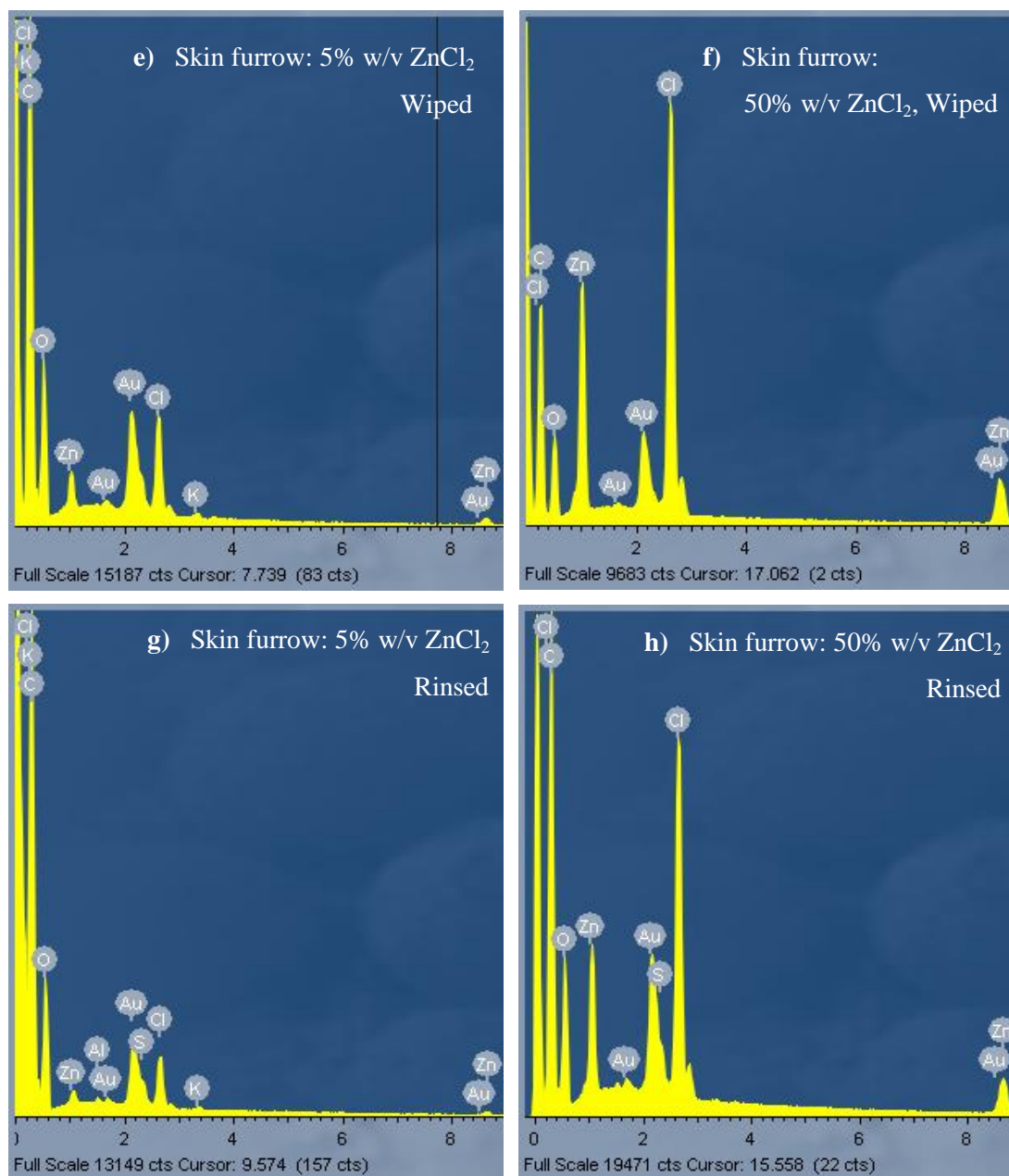
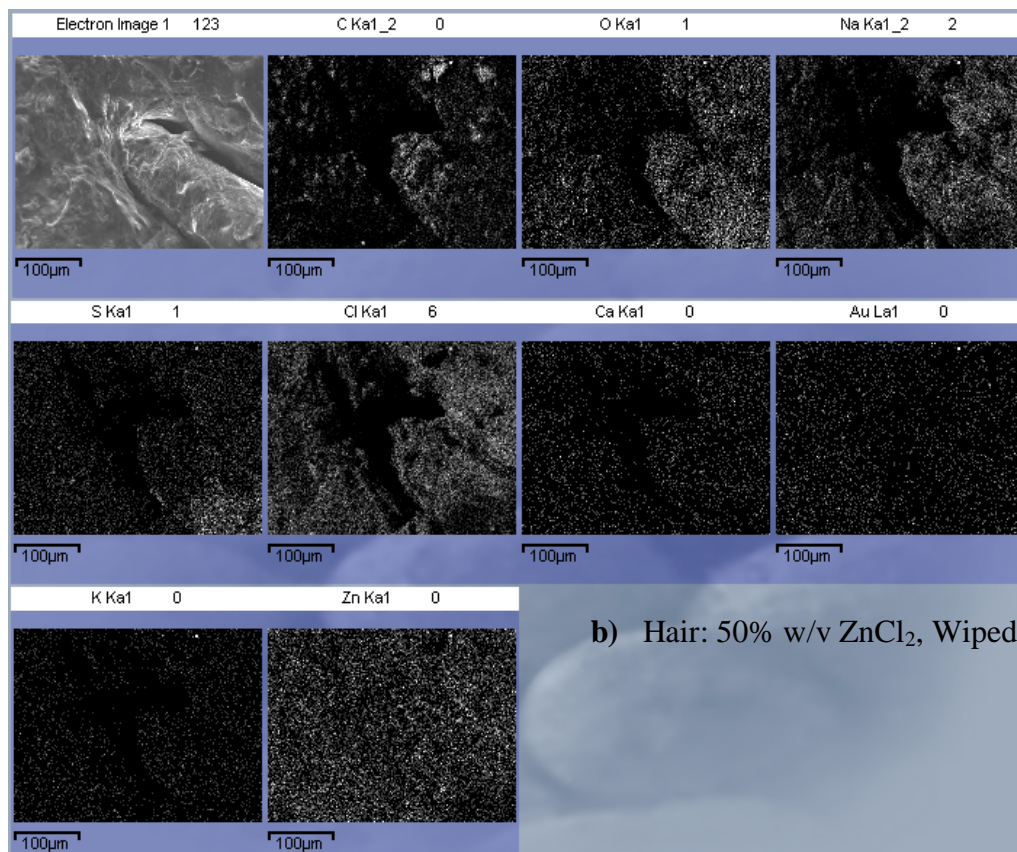
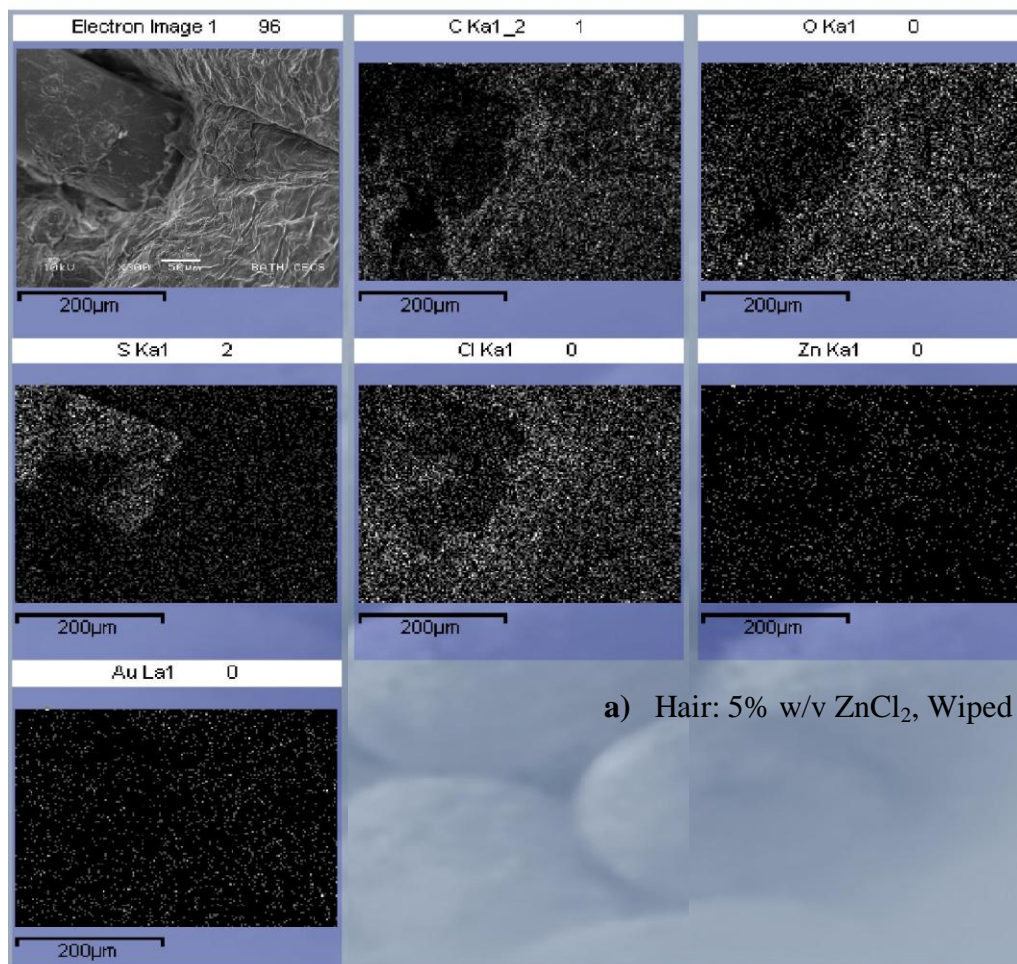
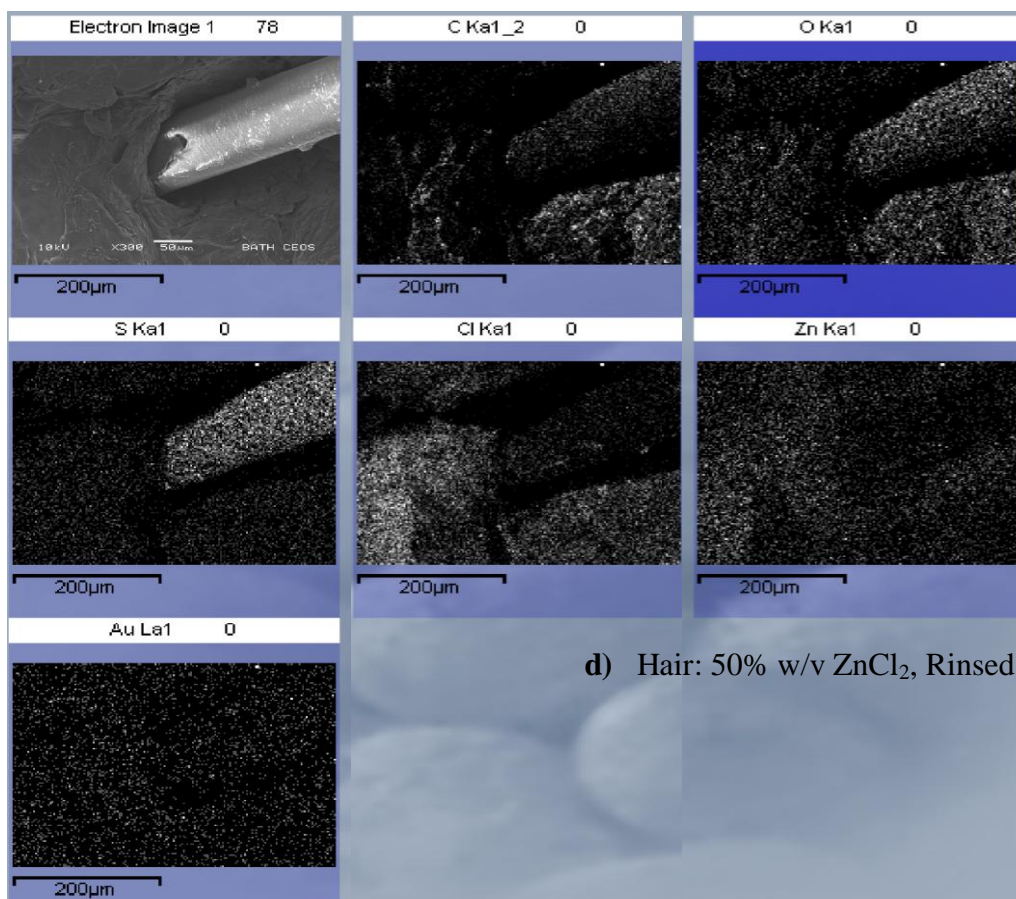
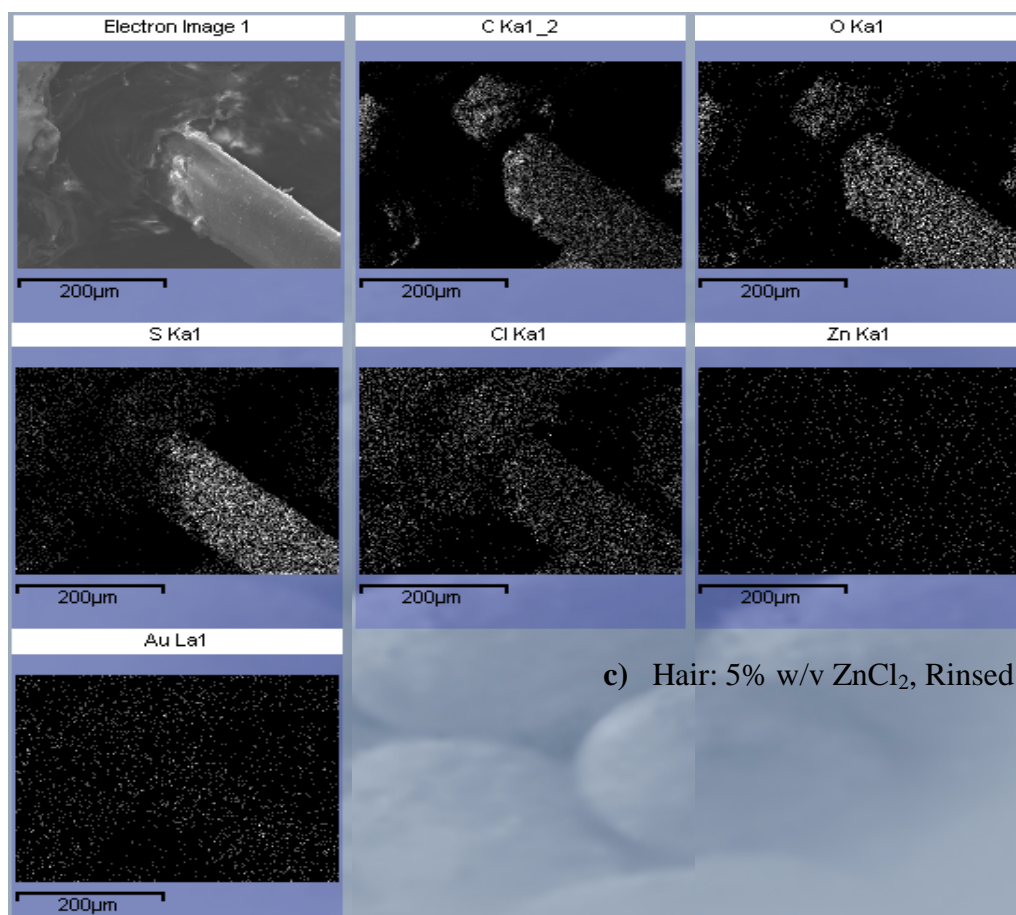


Fig. 4 Energy dispersive X-ray (EDX) spectra of the hair and skin furrows of skin treated with 5% and 50% w/v  $\text{ZnCl}_2$  and dry wiped, or rinsed with deionised water after treatment

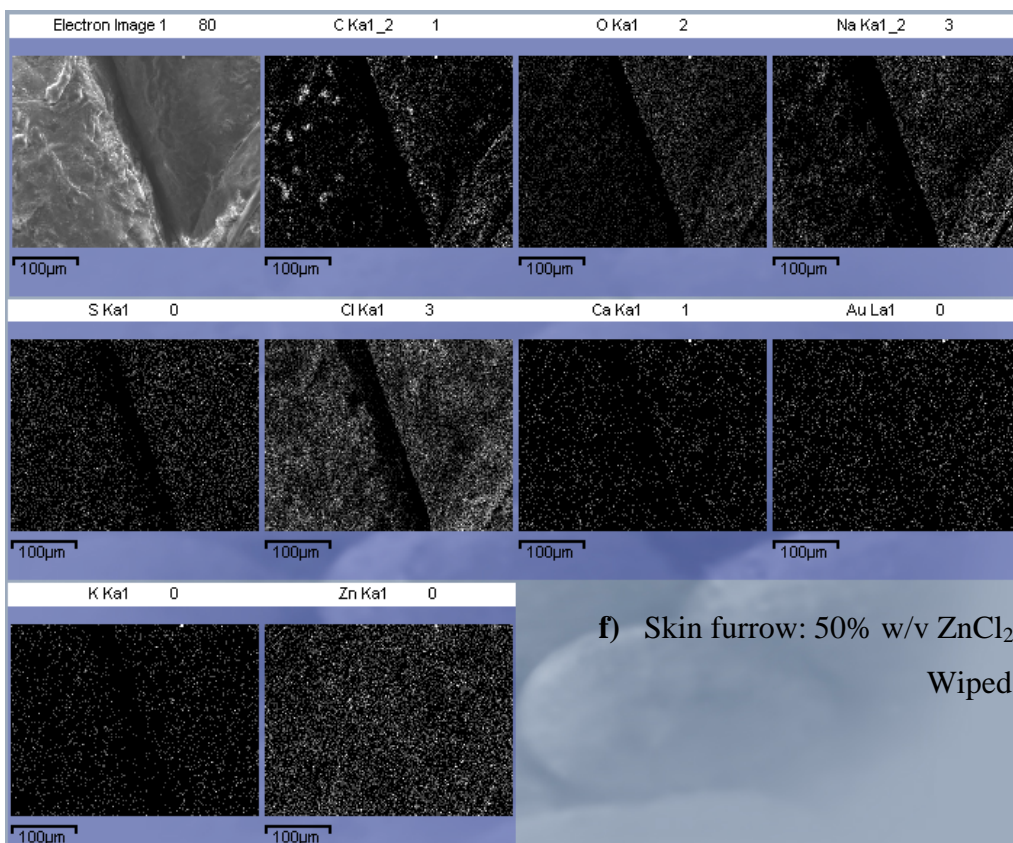
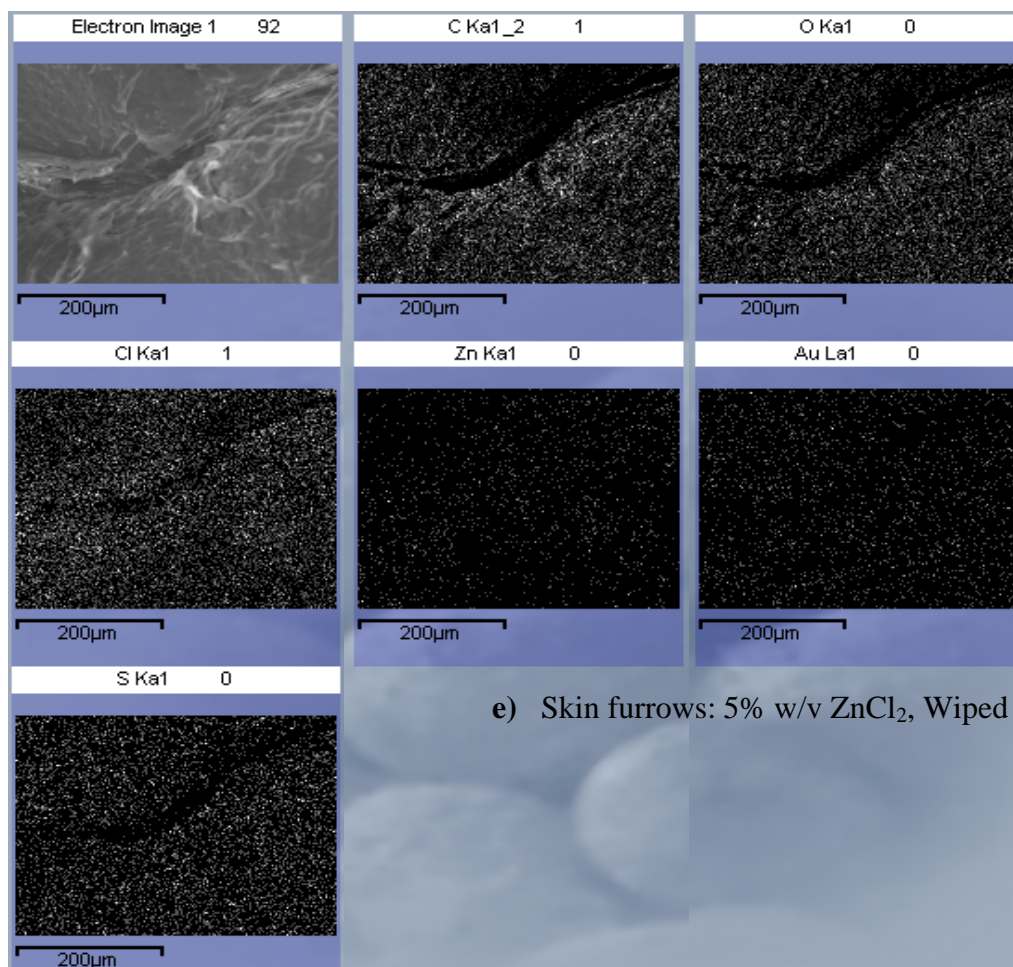
Element mapping showed the distribution and density of each element present on the surface of the skin (Fig. 5). Higher levels of zinc were observed after application of 50% w/v  $\text{ZnCl}_2$  than those following treatment with 5% w/v  $\text{ZnCl}_2$ . In general, rinsing the skin with deionised water reduced the amounts of zinc present on the skin. The element maps indicate that zinc had equal affinity for hair and skin. Chlorine delivered from the  $\text{ZnCl}_2$  was also observed in quantities correlated with the concentrations applied.











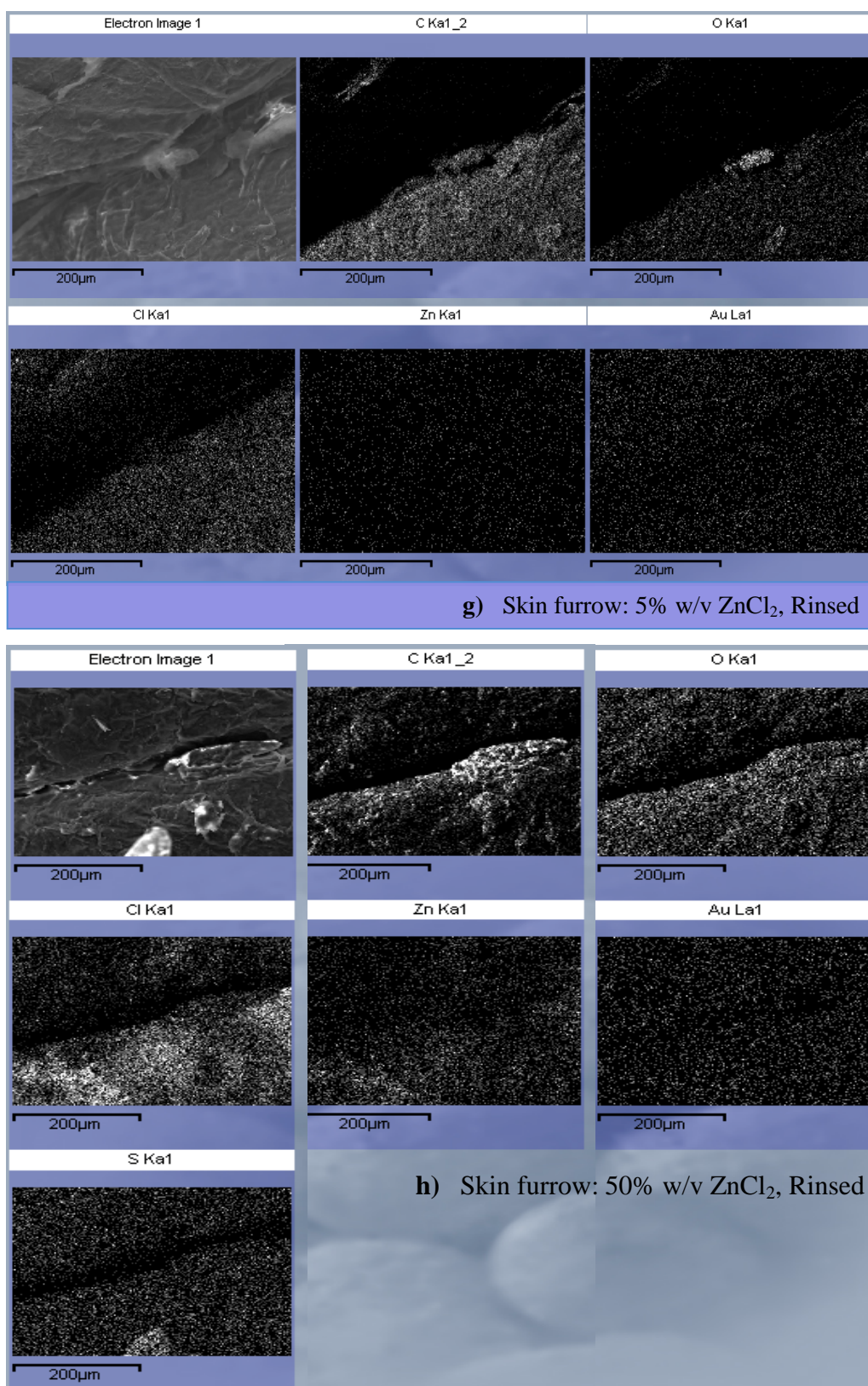


Fig. 5 Element maps showing the distribution of different elements on the skin surface following treatment with 5 % and 50% w/v  $\text{ZnCl}_2$  and then cleaned either with absorbent tissue, or rinsed under deionised water. Samples a-d show areas of the skin with hair, panels e-h show furrows of the skin.

Smooth and rough skin areas

Fig. 6 shows images of smooth and rough areas of skin after treatment with 5 and 50%  $\text{ZnCl}_2$  and subjected to the different cleaning methods described previously. No obvious visual difference was observed between skin samples after treatment with 5% and 50%  $\text{ZnCl}_2$ , nor between cleaning methods.

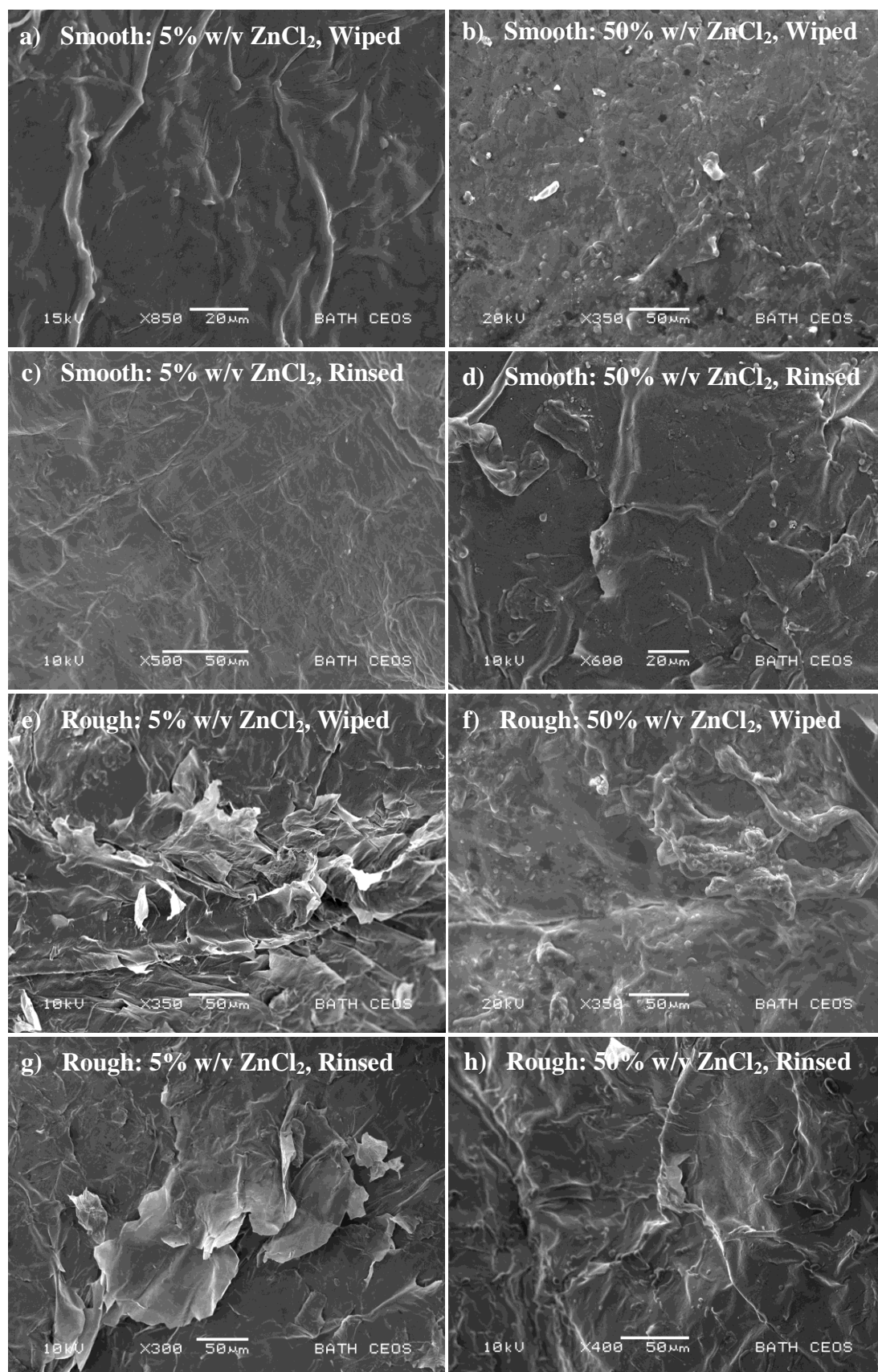
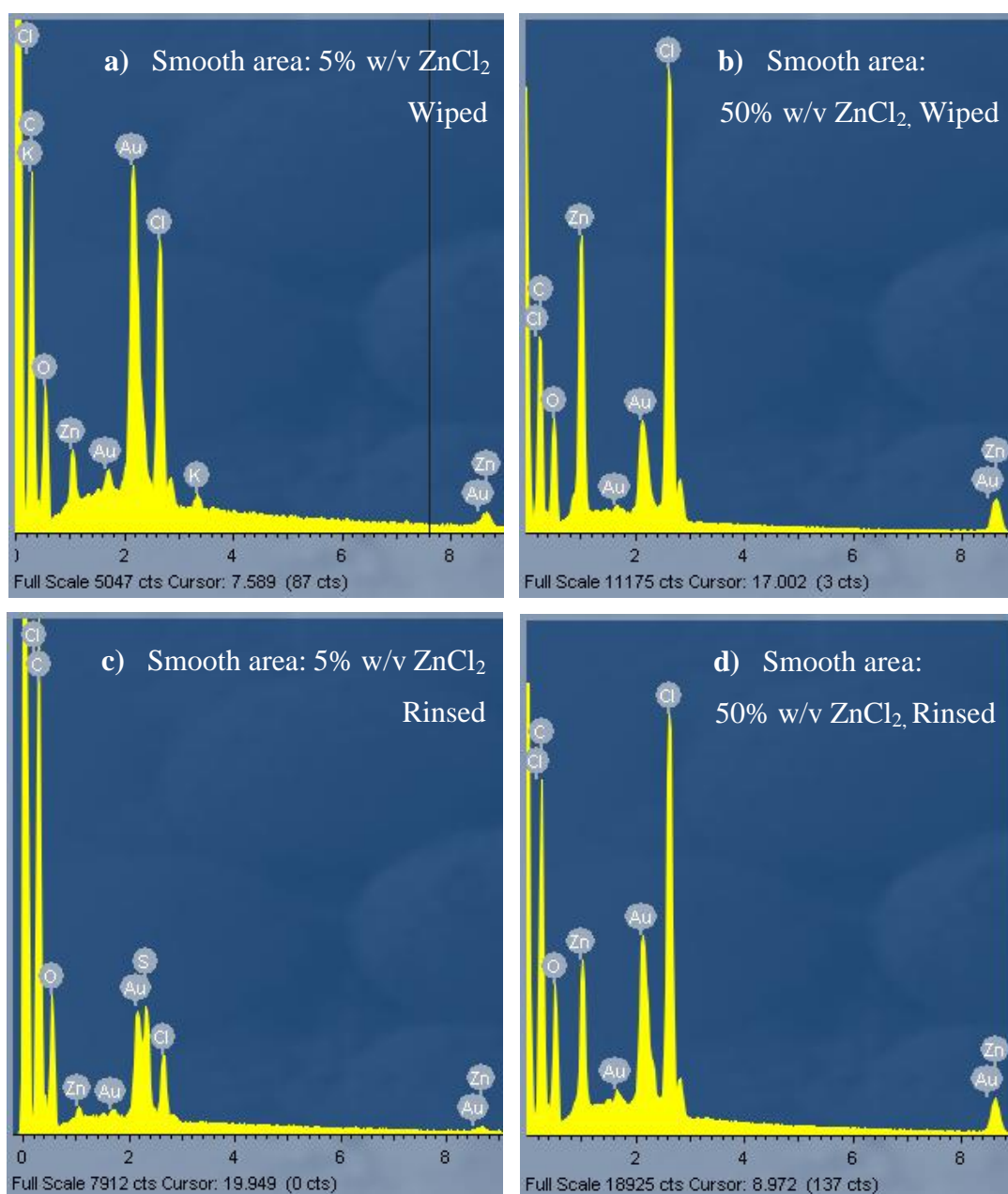


Fig. 6 SEM Images of smooth and rough skin areas. Left and right panels were treated with 5% and 50% w/v ZnCl<sub>2</sub>, respectively. Samples a), b), e), and f), were wiped dry directly after treatment, and samples c), d), g) and h) were rinsed under deionised water before drying.



Spectra of smooth and rough skin areas showed that levels of zinc detected was dependent on the concentration applied and the type of cleaning method utilised after passive diffusion; i.e., the more concentrated  $\text{ZnCl}_2$  solution delivered more zinc to the skin which was more efficiently removed by washing with deionised water (Fig.7). While the zinc cannot be quantified in the images, qualitative comparisons are possible. The amounts of zinc detected on smooth and rough surfaces appeared were similar for the same donor concentrations (Figs. 7a, e, b, f). Rinsing the skin with deionised water clearly removed zinc from the skin surface (Figs. 7c, d, g, h).



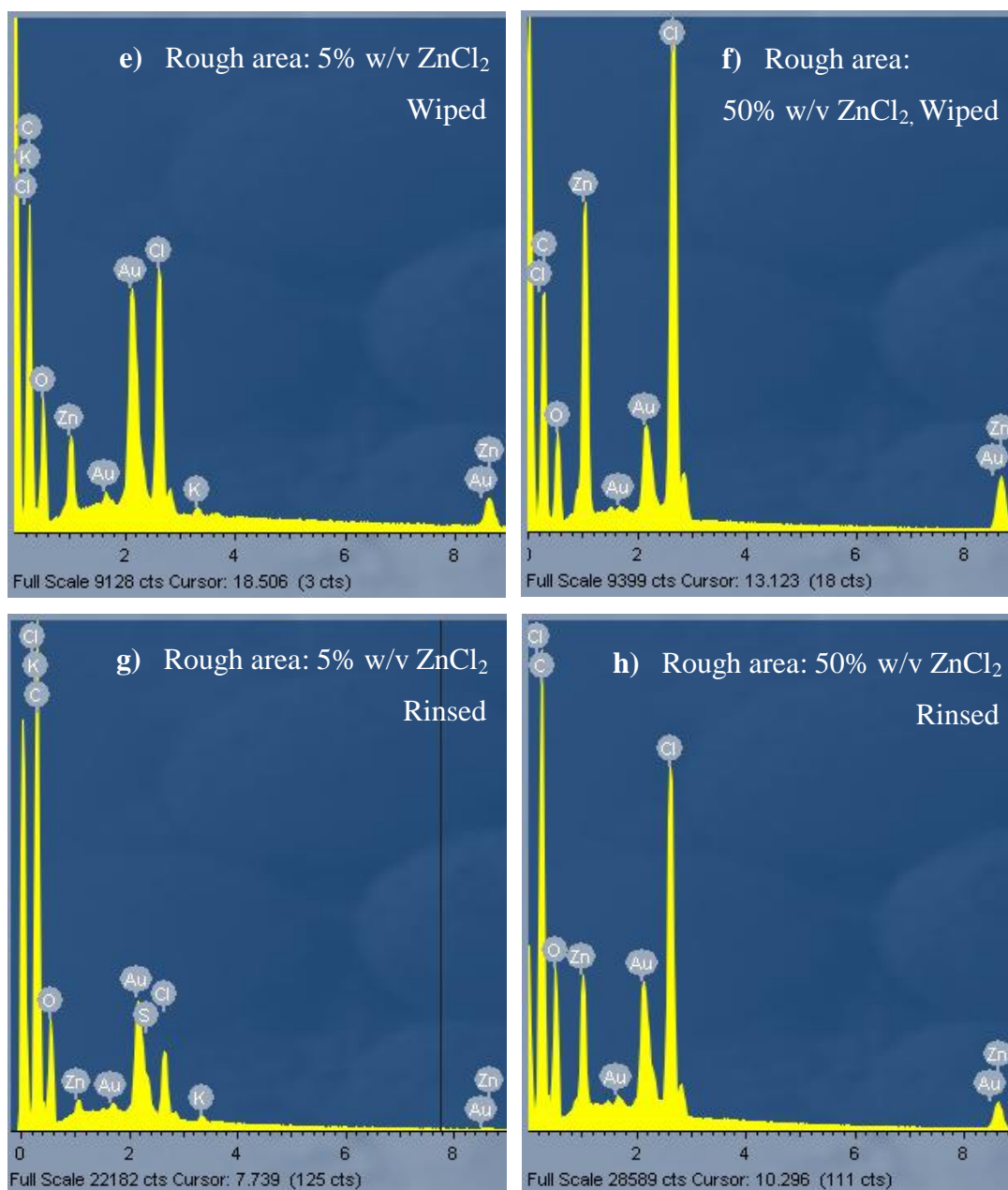
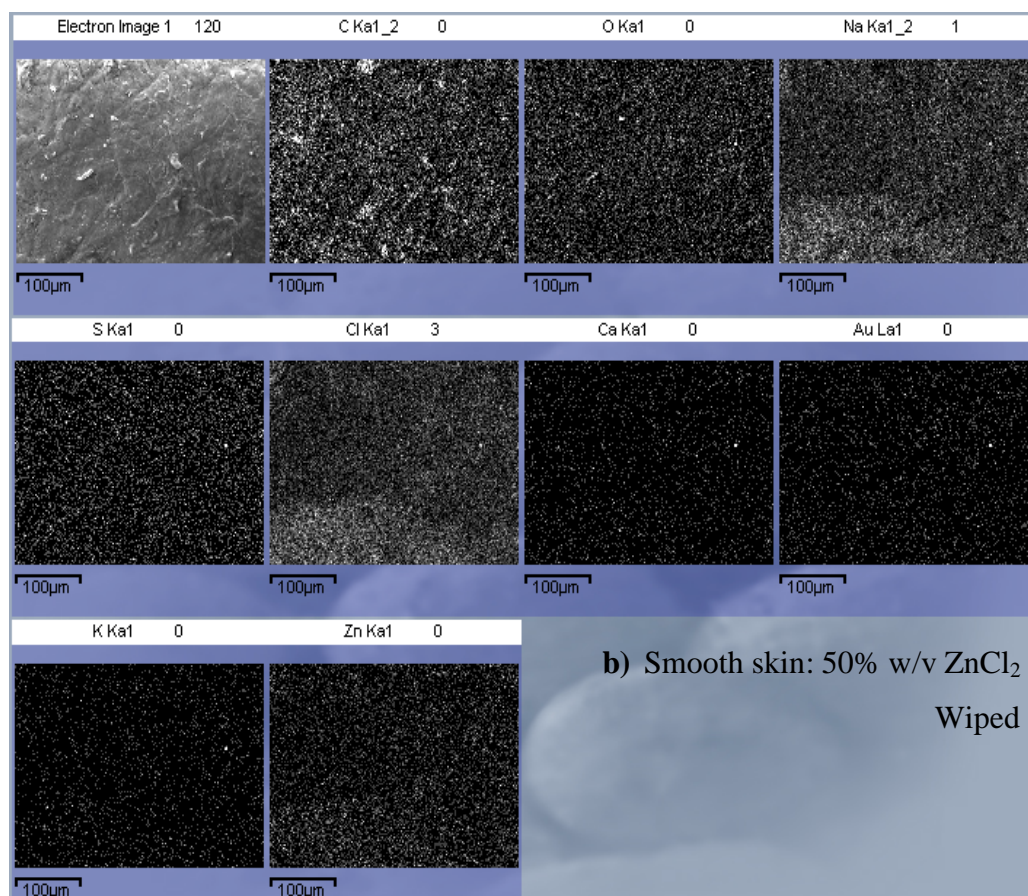
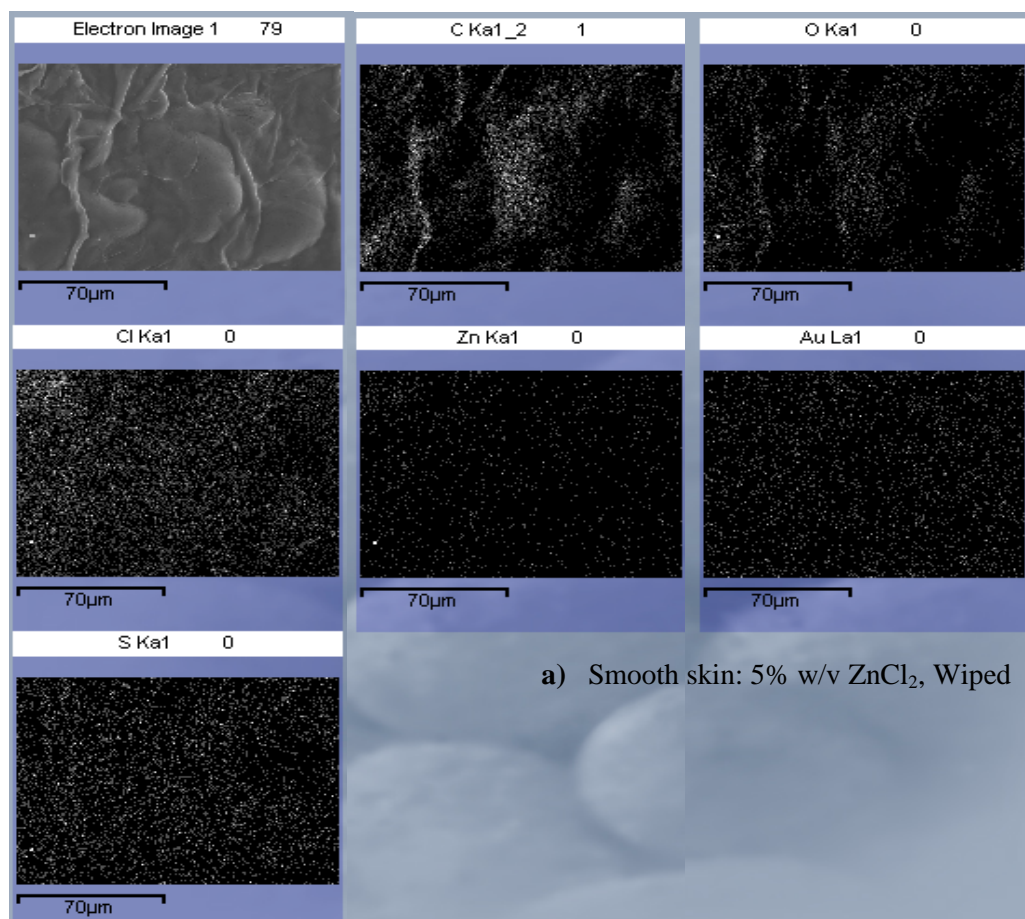
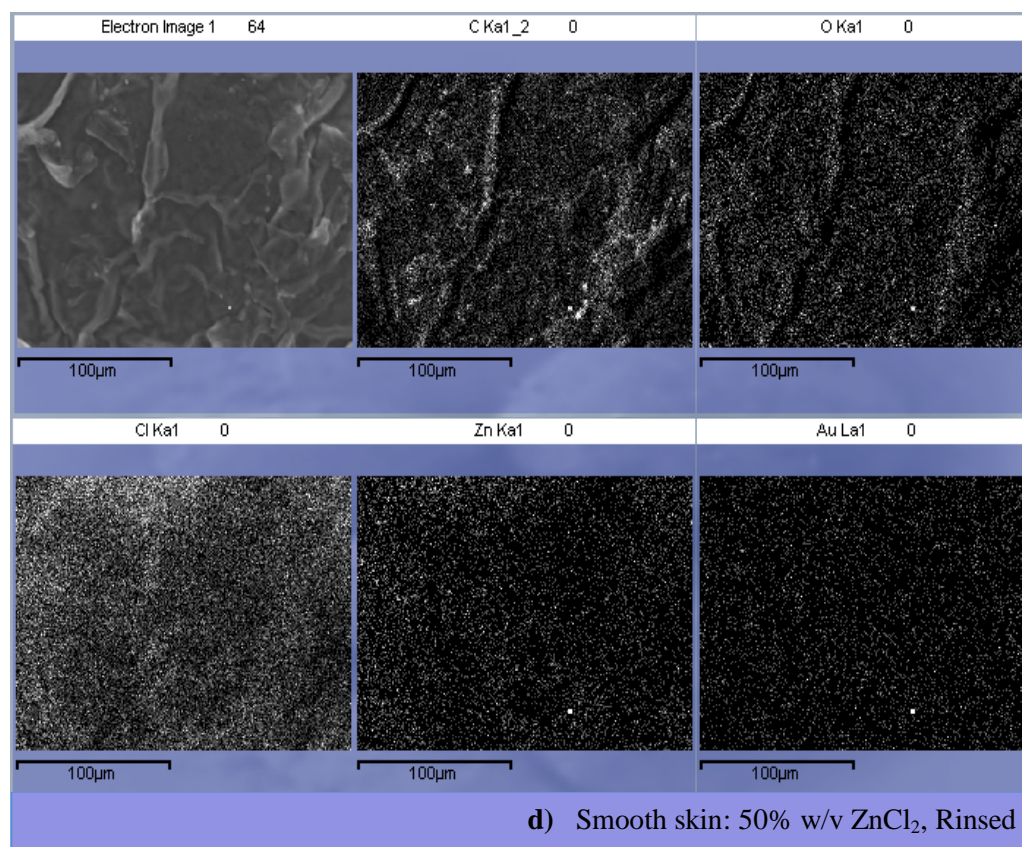
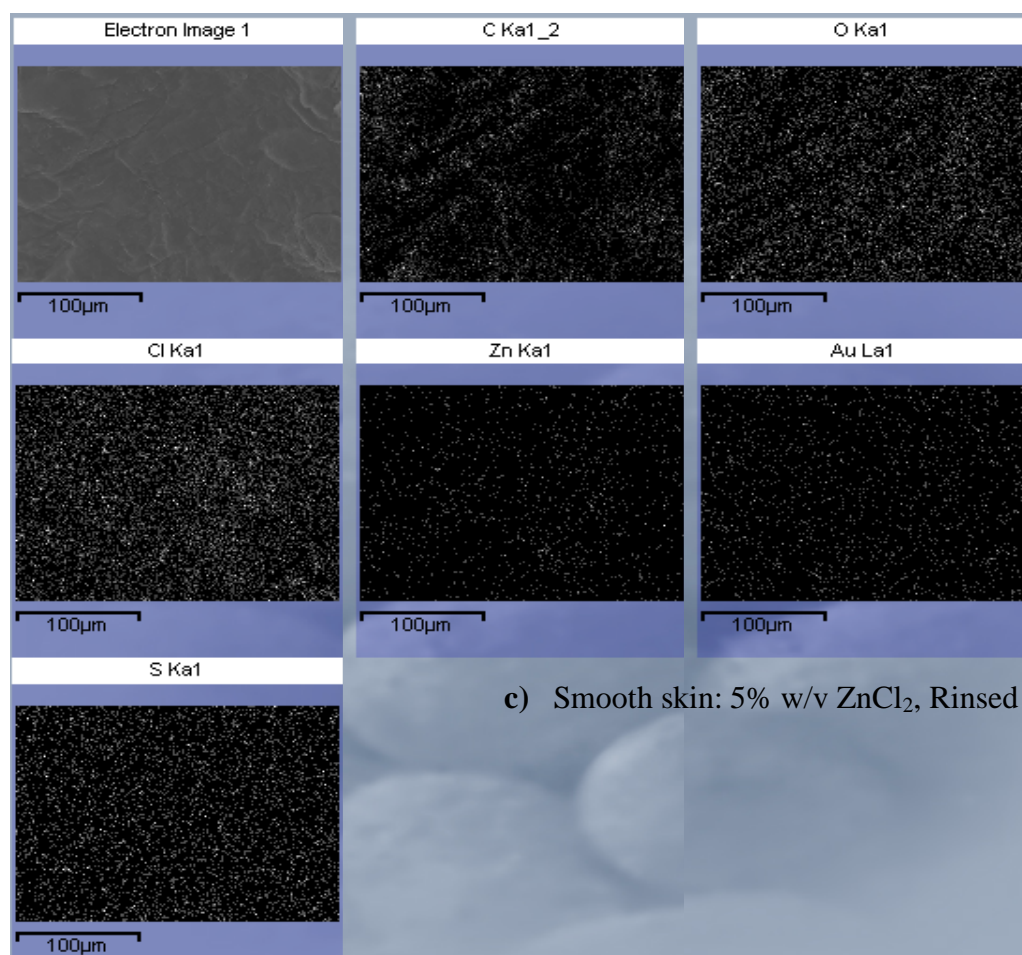


Fig. 7 EDX spectra of the hair and skin furrows of skin treated with 5 and 50% w/v  $\text{ZnCl}_2$  and wiped, or rinsed with deionised water after treatment

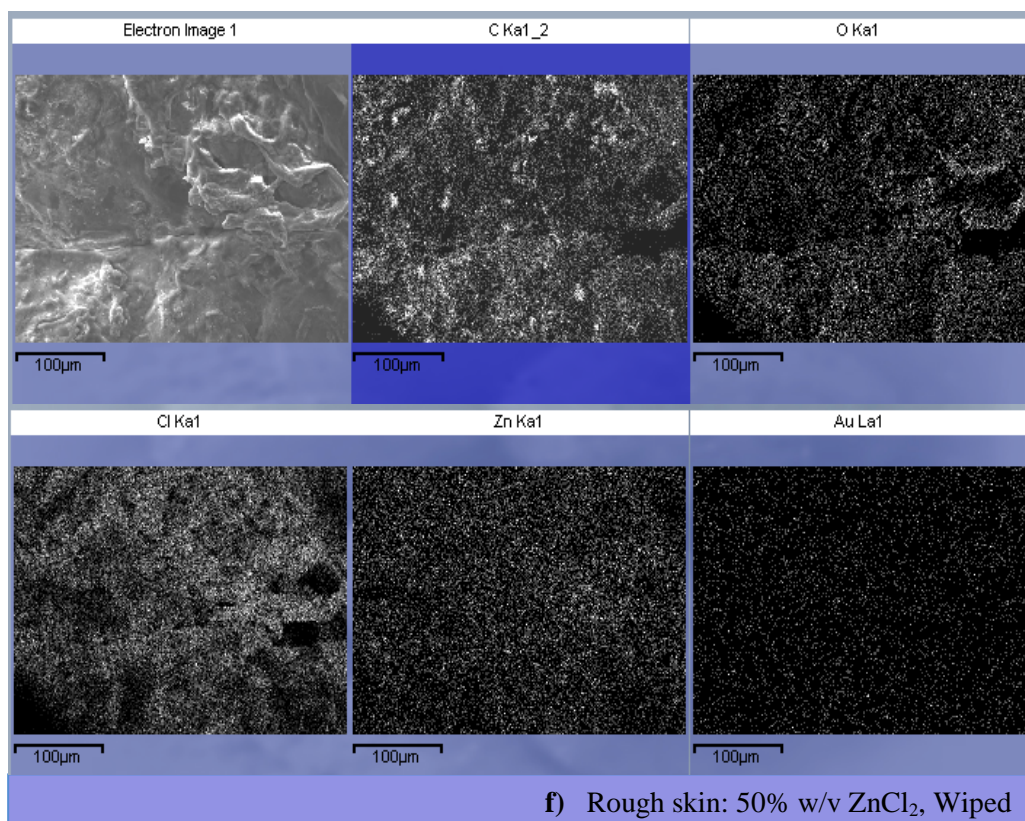
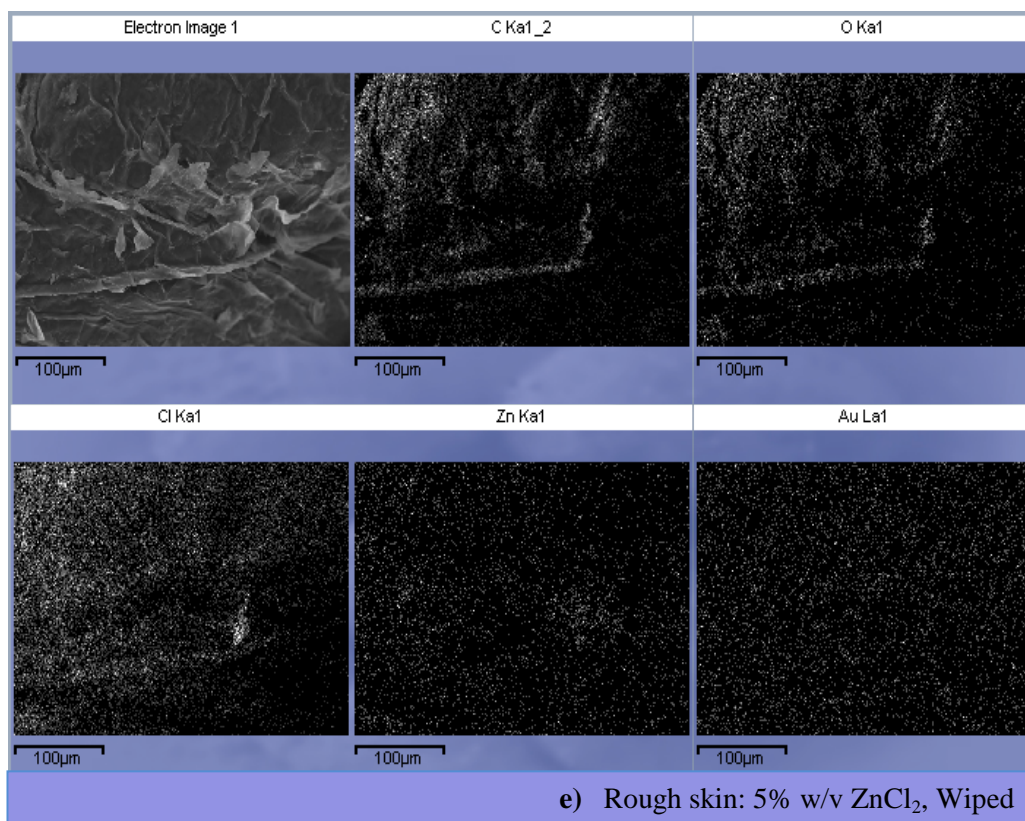
The presence and levels of zinc on the skin were confirmed by element maps (Fig. 8). Higher levels of zinc were observed on skin treated with 50% w/v  $\text{ZnCl}_2$  compared to 5% w/v  $\text{ZnCl}_2$ . However, no apparent differences related to the skin cleaning process were observed.











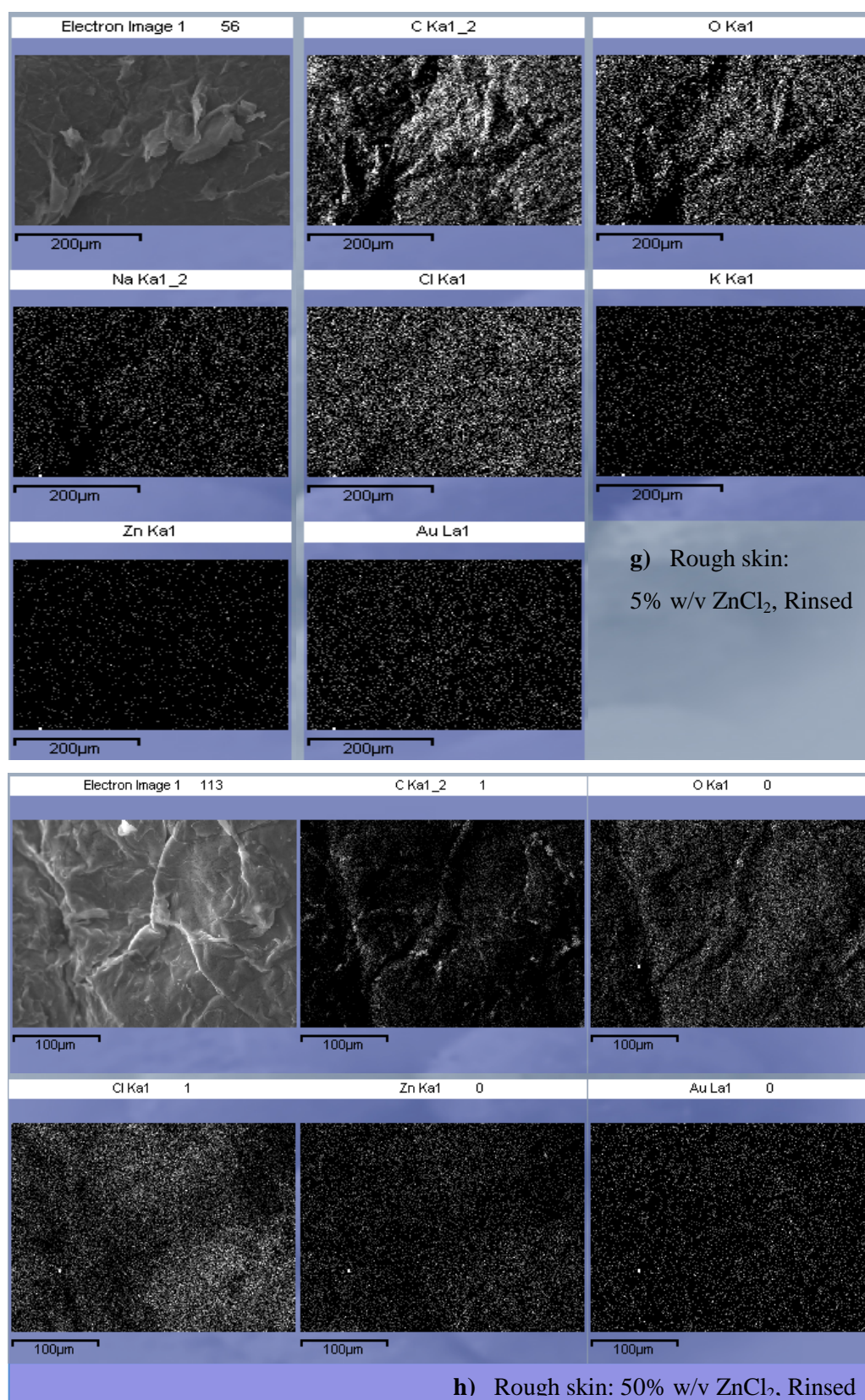


Fig. 8 Element maps showing the distribution of different elements on smooth and rough skin surfaces treated with 5 % and 50% w/v  $\text{ZnCl}_2$  and then cleaned either with absorbent tissue, or rinsed under deionised water. Samples a-d, and e-h show smooth and rough areas of skin, respectively.

### **Results - Part 3**

#### **Comparison of zinc delivery from various formulations**

Delivery of zinc to the skin from 2% Zn lactate (20:80 and 80:20 gly/H<sub>2</sub>O), 5% and 50% ZnCl<sub>2</sub> and Morhulin<sup>®</sup> (38% ZnO) was investigated. Excess formulation was removed either by rinsing the skin with deionised water or, in the case of Morhulin<sup>®</sup>, with isopropyl alcohol wipes.

#### **Hair**

Fig. 9a-f show hairs on the skin surface after treatment with the different zinc preparations. With the exception of skin treated with Morhulin<sup>®</sup> (Fig. 9e) (which appeared to leave a thin white coating on the hair and surrounding areas of skin), there was no obvious visual difference between the formulations; no deposits, degradation of the skin or hair, or discolouration, were observed.

EDX spectra of the hairs and surrounding areas showed no zinc detected on the control (H<sub>2</sub>O), or 2% w/v Zn lactate treated skin (Fig. 10 a-c). The absence of zinc was confirmed in corresponding element maps of the skin surfaces (Fig. 11a-b). A small zinc peak was observed in Fig 10d, which corresponded to skin treated with 5% w/v ZnCl<sub>2</sub>. Larger peaks were present in spectra from skin treated with Morhulin<sup>®</sup> (38% w/w ZnO) and 50% w/v ZnCl<sub>2</sub> (Fig. 10e-f). Where concentrations were sufficient, small secondary zinc peaks were visible at 8.6 keV (Fig. 10d-f). Background levels of chlorine (thought to be naturally occurring) was observed in Figs. 10a- c, e. Larger chlorine peaks (approximately twice the size of the zinc peaks) were observed in Fig. 10d, f with ZnCl<sub>2</sub>. The corresponding element maps also detected higher levels of chlorine (Fig. 11c, e).

Calcium and traces of aluminium of unknown origin were detected in the spectrum of the control (Fig. 10a). A small sodium peak was seen in the spectrum of 2% Zn lactate (80:20, gly/H<sub>2</sub>O) treated skin (Fig. 10c) which was not present in the element map possibly due to the concentration falling below the limit of detection (Fig. 11b). Sulphur was also present in each EDX spectrum (Fig.9 a-h) and was concentrated in the hairs (Fig. 11).

The EDX spectra concur with the element maps in that no zinc was detected in the control and Zn lactate treated samples (Fig. 11 a, b) whereas zinc was detected in 5%, 50% w/v



ZnCl<sub>2</sub> and Morhulin<sup>®</sup> treated skin and that the levels detected increased with the increasing zinc concentration applied (Fig. 11c-e).

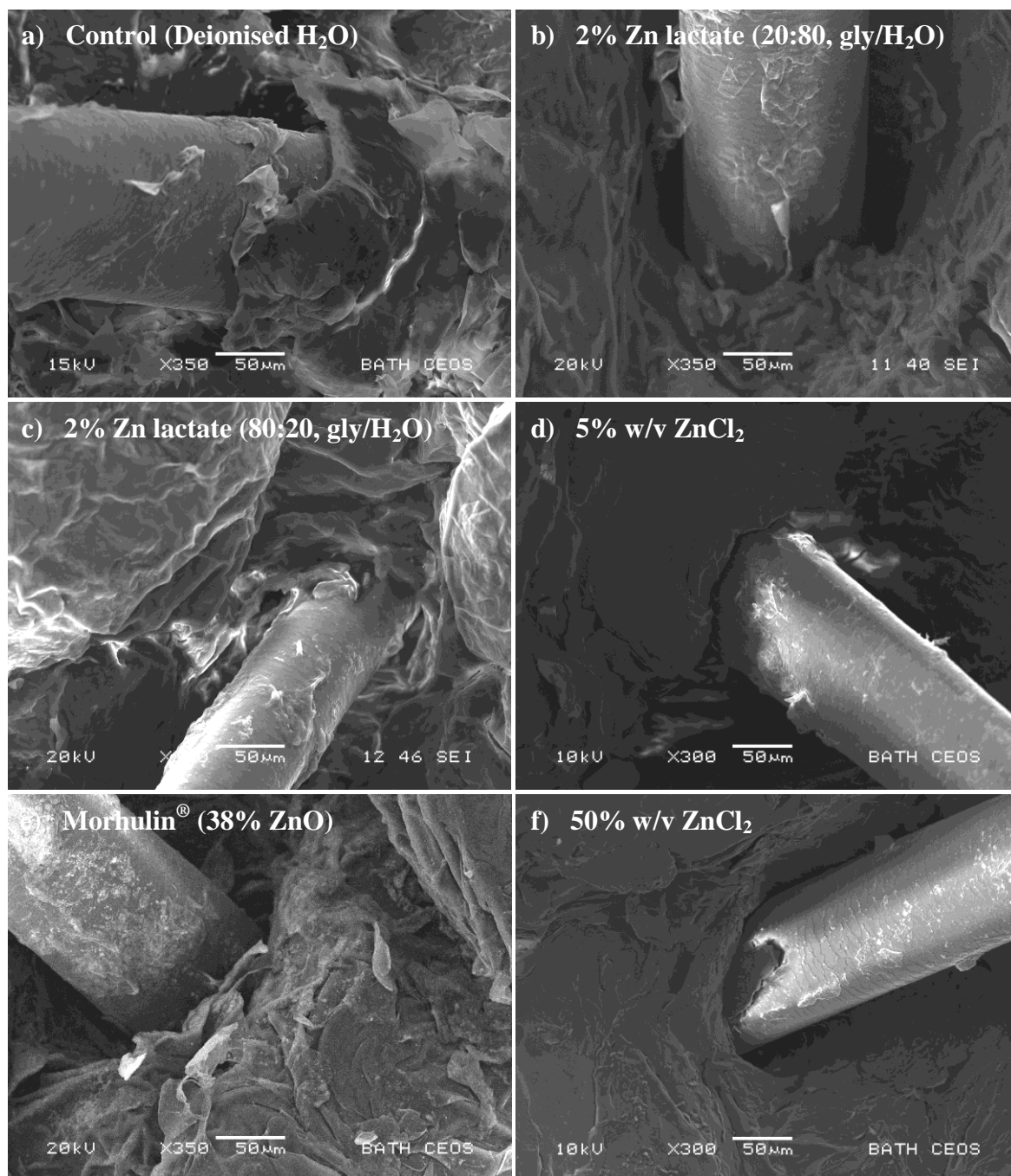


Fig. 9 SEM images of hairs on the surface of the skin: panels (a) through (f) represent treatment with deionised water (control), 2% w/v zinc lactate (20:80, gly/H<sub>2</sub>O), 2% w/v zinc lactate (80:20, gly/H<sub>2</sub>O), 5% w/v ZnCl<sub>2</sub>, Morhulin<sup>®</sup>, and 50% w/v ZnCl<sub>2</sub>, respectively.

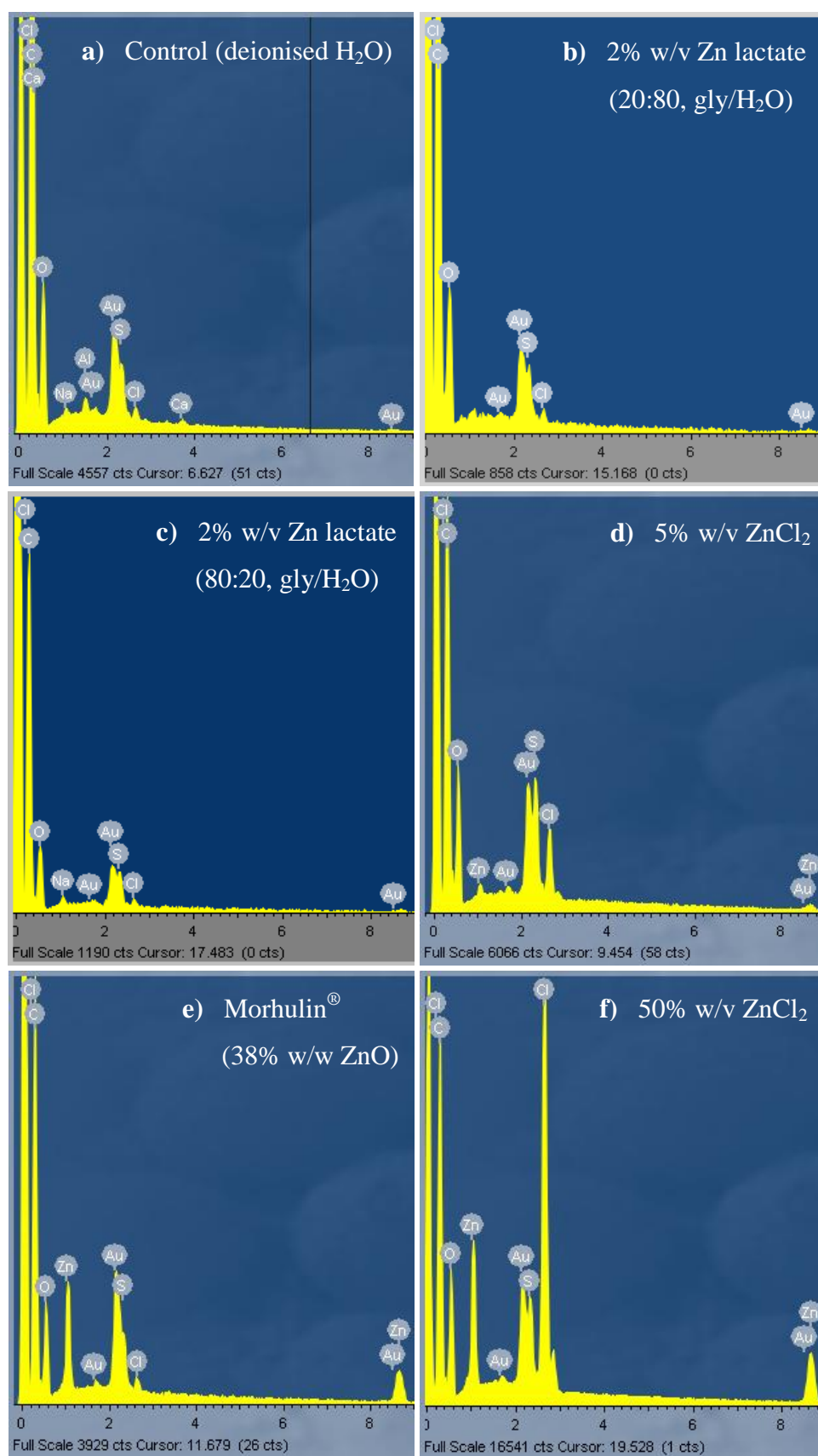
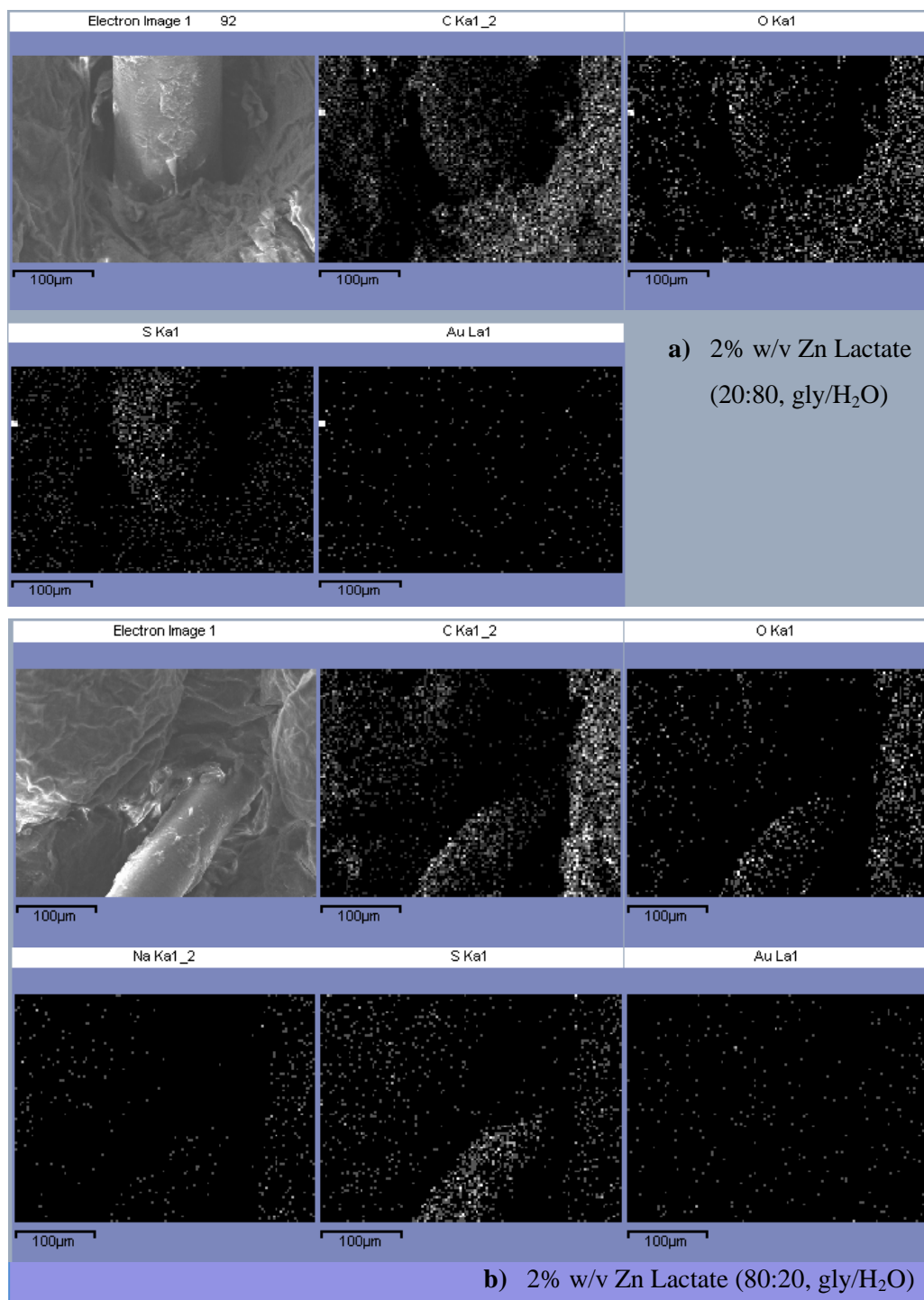
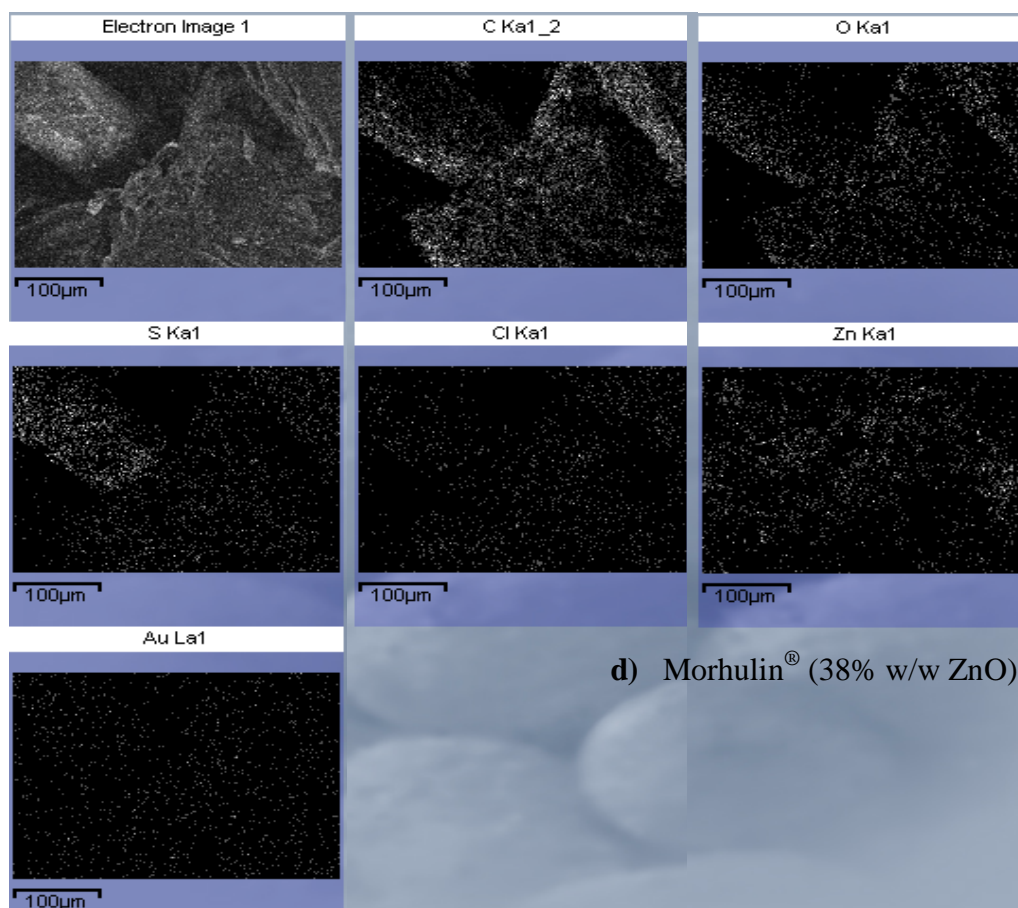
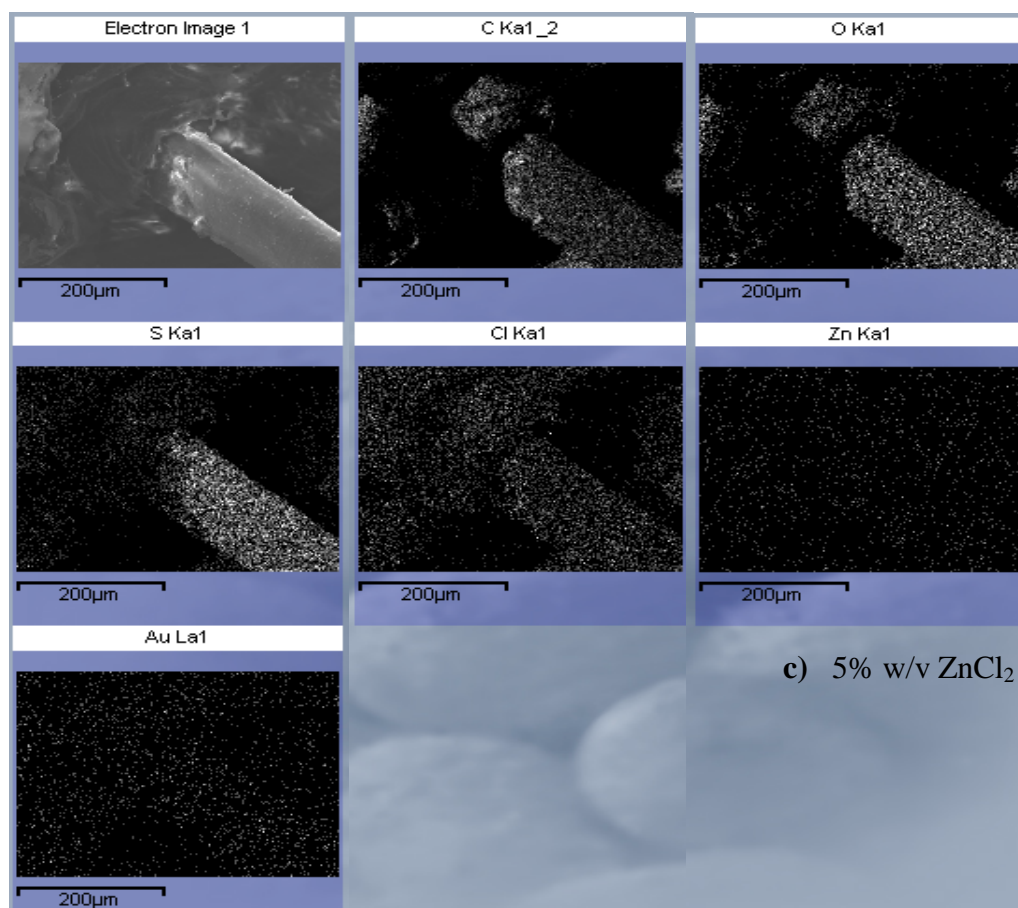


Fig.10 EDX spectra of hairs and surrounding skin areas following application of a) deionised water (control), b) 2% w/v zinc lactate (20:80, gly/H<sub>2</sub>O), c) 2% w/v zinc lactate (80:20, gly/H<sub>2</sub>O), d) 5% w/v ZnCl<sub>2</sub>, e) Morhulin<sup>®</sup>, and f) 50% w/v ZnCl<sub>2</sub>.







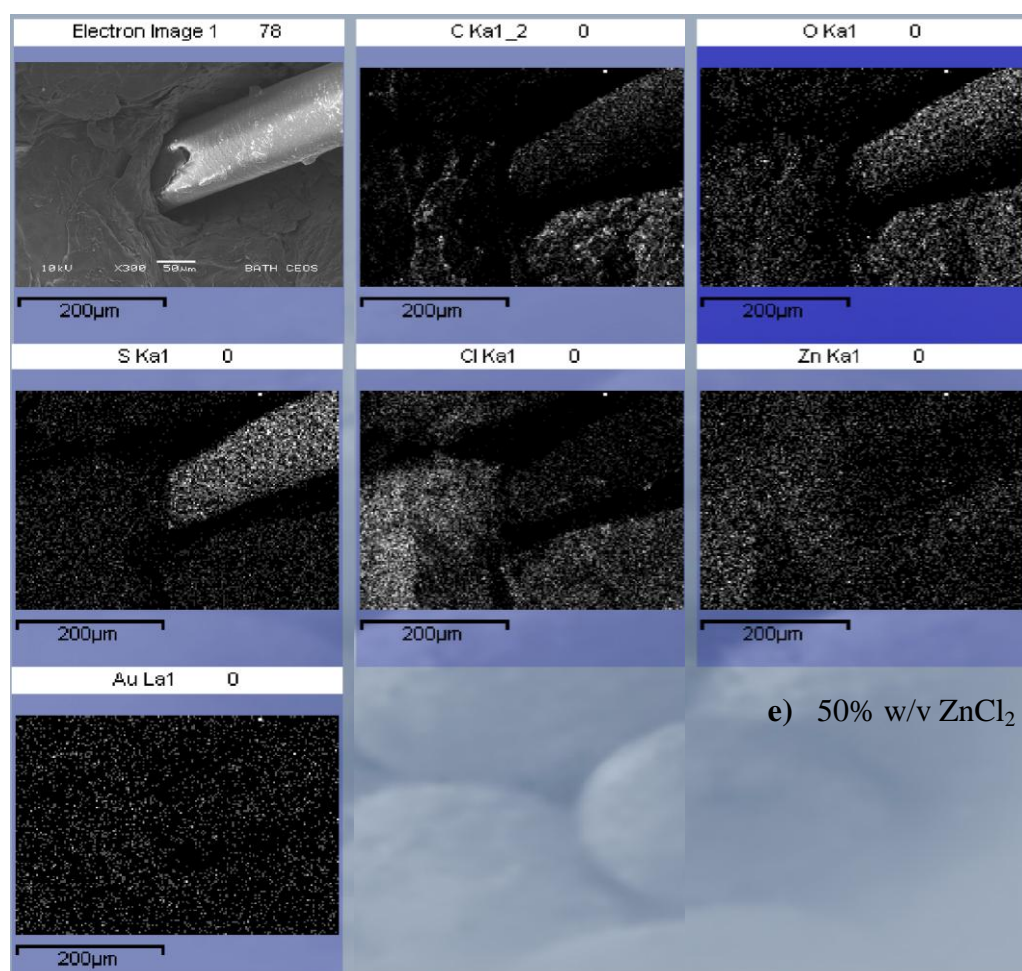


Fig. 11 Element maps showing the distribution of different elements on and around hairs following application of a) deionised water (control), b) 2% w/v zinc lactate (20:80, gly/H<sub>2</sub>O), c) 2% w/v zinc lactate (80:20, gly/H<sub>2</sub>O), d) 5% w/v ZnCl<sub>2</sub>, e) Morhulin<sup>®</sup>, and f) 50% w/v ZnCl<sub>2</sub>.

### Skin furrows

Images of skin furrows post-treatment are shown in Fig. 12. No visible signs of deposits or changes in structure were observed on skin treated with deionised water (control), 2% w/v Zn lactate and 5% w/v ZnCl<sub>2</sub> (Fig. 12a-d). On closer inspection, Morhulin<sup>®</sup> treated skin appeared to be coated with a thin white film of deposit (Fig. 12e). Skin treated with 50% w/v ZnCl<sub>2</sub> looked slightly drier than other samples (Fig. 12f).

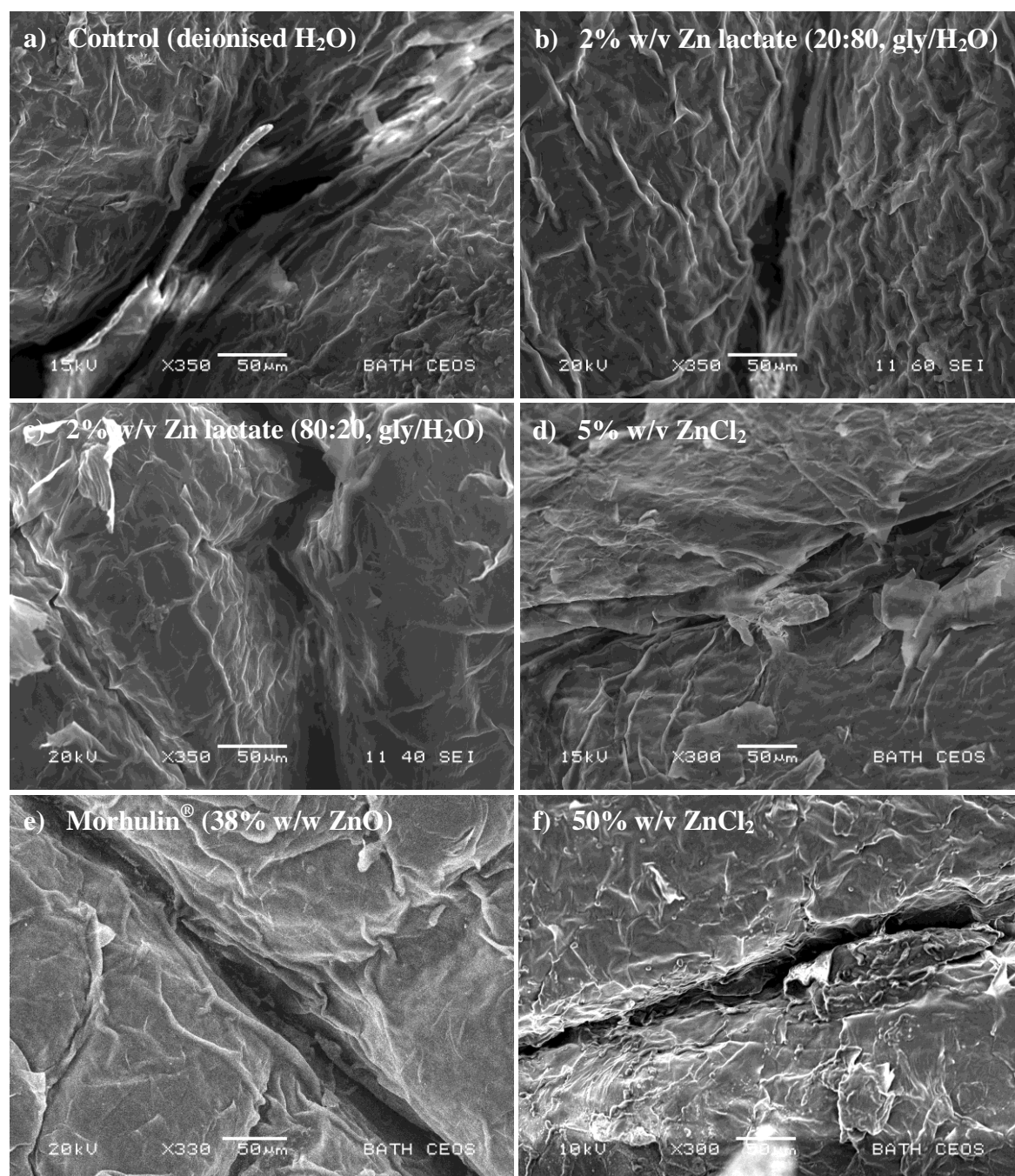


Fig. 12 SEM images of furrows on the skin surface. Panels (a) through (f) represents treatment with deionised water (control), 2% w/v zinc lactate (20:80, gly/H<sub>2</sub>O), 2% w/v zinc lactate (80:20, gly/H<sub>2</sub>O), 5% w/v ZnCl<sub>2</sub>, Morhulin®, and 50% w/v ZnCl<sub>2</sub>, respectively.

EDX spectra of skin furrows are shown in Fig. 13. No zinc was detected in the control or 2 % w/v zinc lactate (80:20, gly/H<sub>2</sub>O) treated skin (Fig. 13a, c). Small peaks were visible for 2% w/v zinc lactate (20:80, gly/H<sub>2</sub>O) and 5% w/v ZnCl<sub>2</sub> (Fig. 13b, d) treated skin. The main zinc peak seen in Fig. 13e is smaller than that seen in Fig. 10e for Morhulin® (hair) possibly as a result of the additional surface area offered by the hair. The zinc peak for

50% w/v  $\text{ZnCl}_2$  is similar in size to that seen previously in Fig. 10f and is the largest out of all spectra indicating the highest amount of zinc present on the skin.

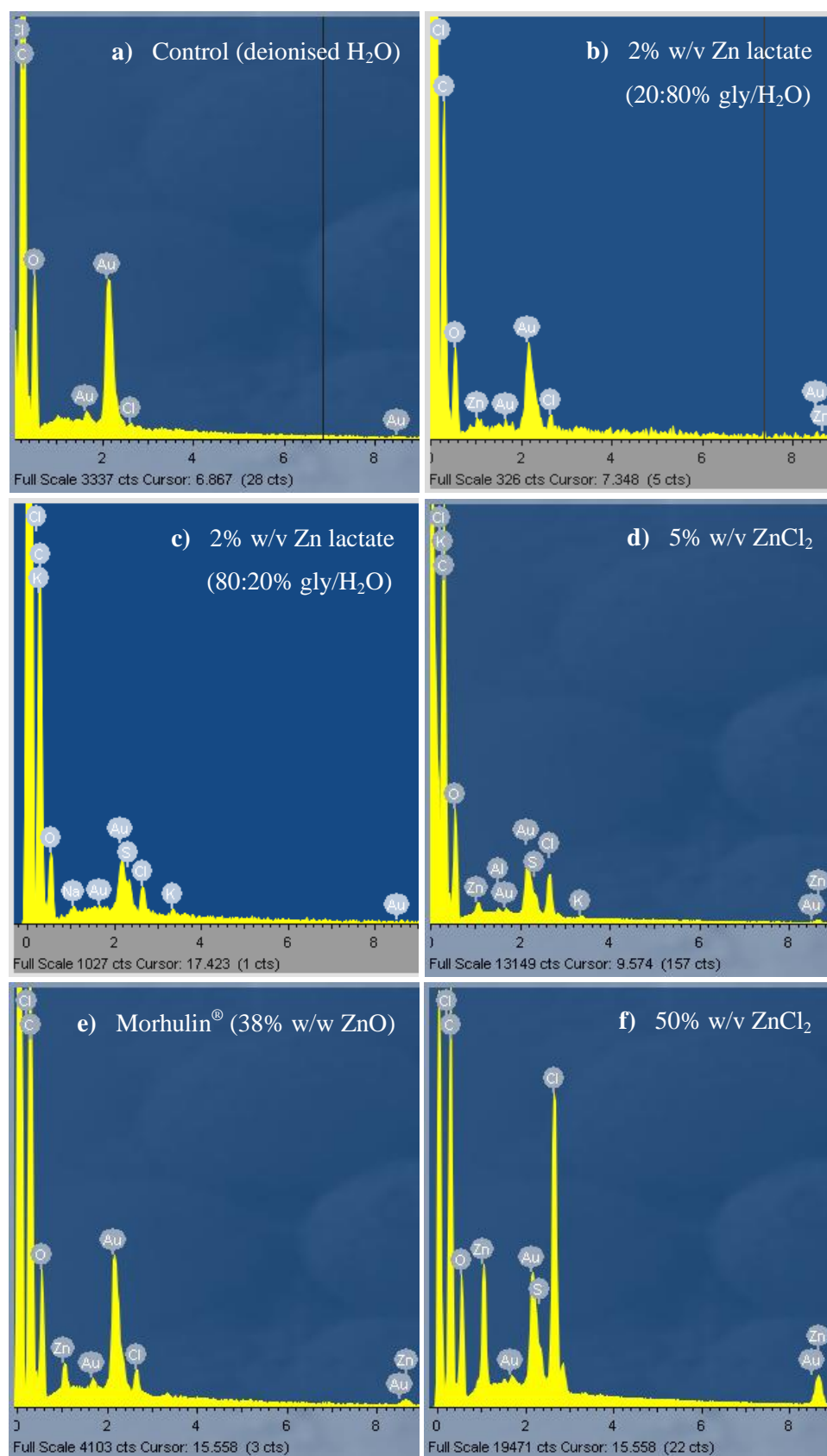
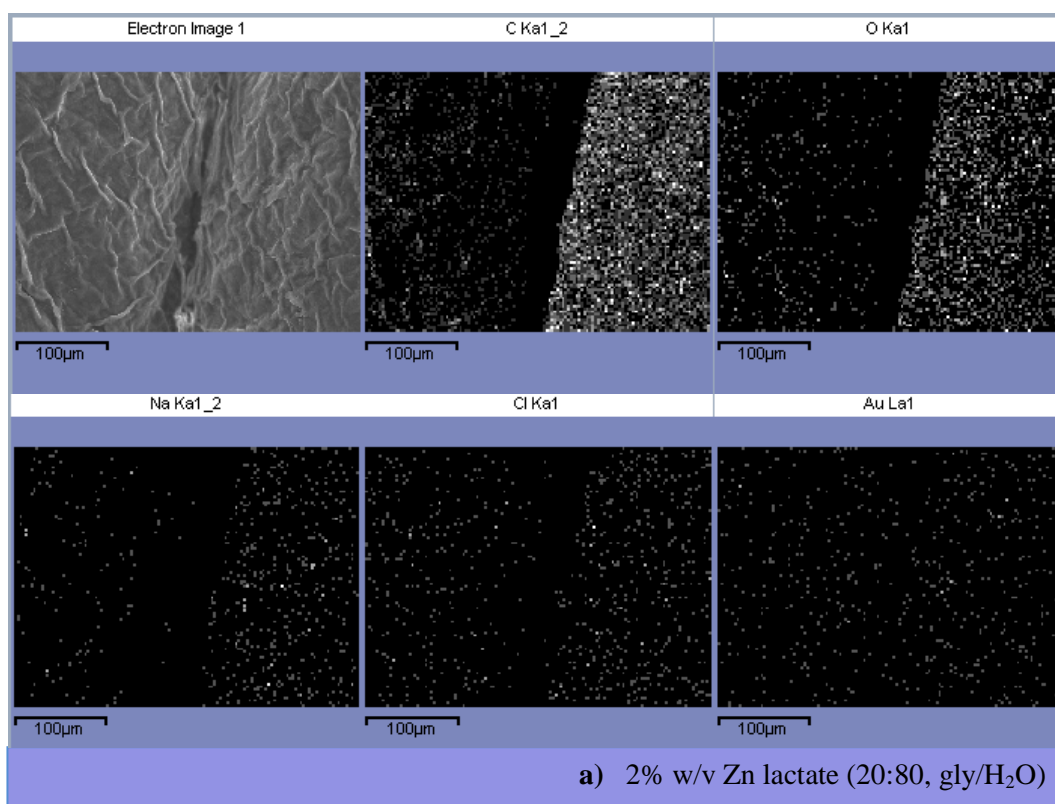


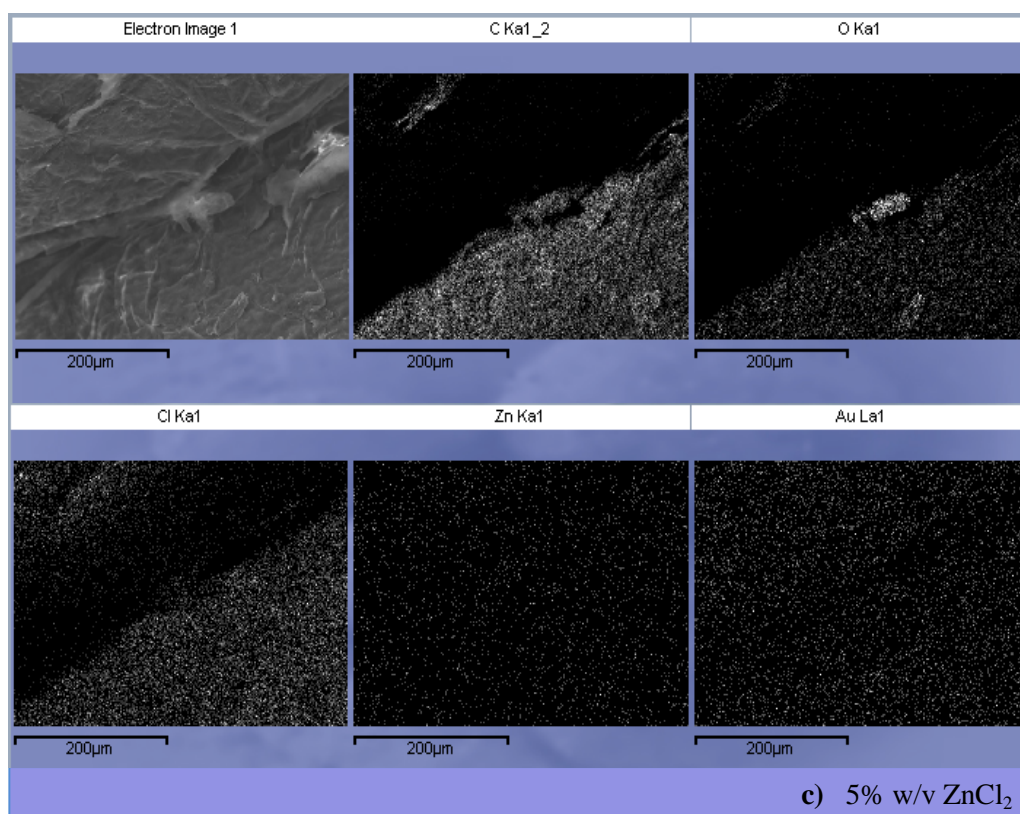
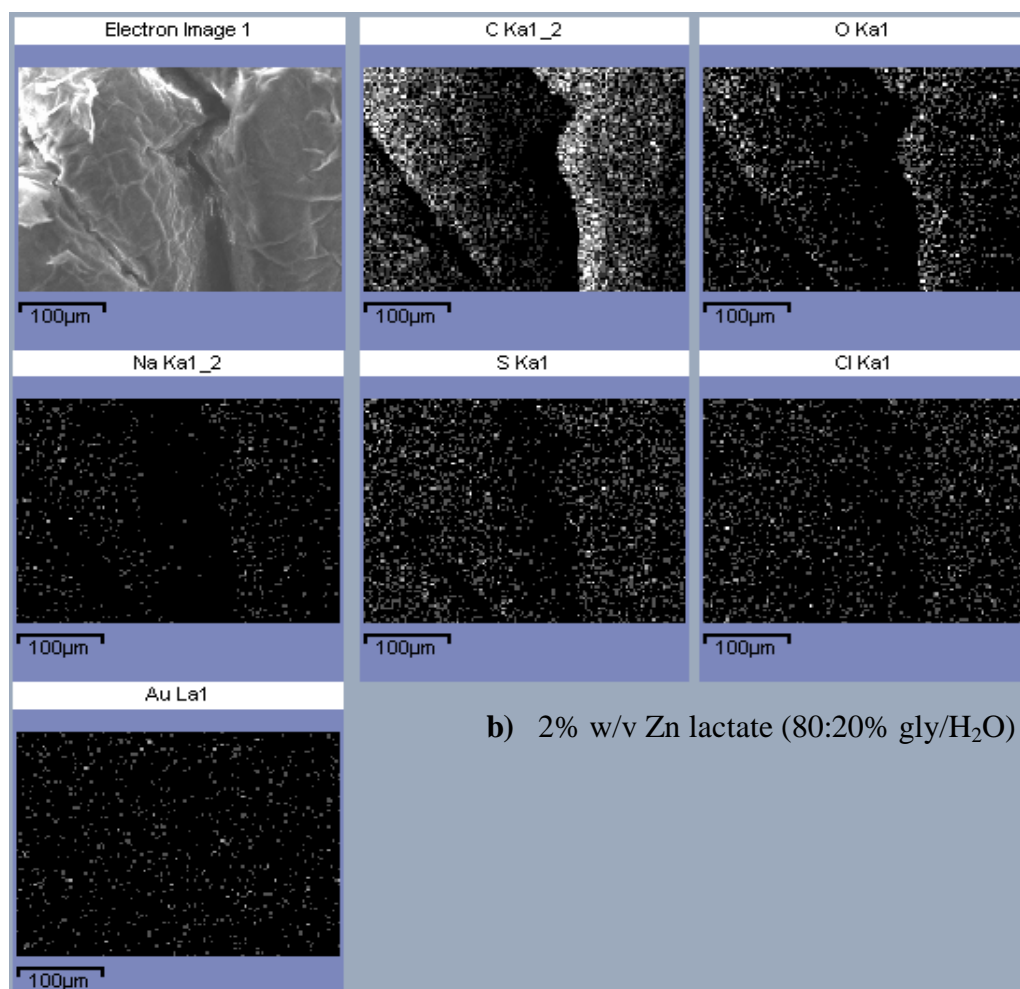
Fig. 13 EDX spectra of furrows following application of a) deionised water (control), b) 2% w/v zinc lactate (20:80, gly/H<sub>2</sub>O), c) 2% w/v zinc lactate (80:20, gly/H<sub>2</sub>O), d) 5% w/v ZnCl<sub>2</sub>, e) Morhulin®, and f) 50% w/v ZnCl<sub>2</sub>.



The EDX spectrum of 2% Zn lactate (20:80, gly/H<sub>2</sub>O) treated skin showed a small peak for zinc (Fig. 13b), which was not present in the element map (Fig. 14a). In agreement with the EDX results, the element map of Zn lactate (80:20, gly/H<sub>2</sub>O) treated skin did not show the presence of zinc (Fig. 14b). Zinc was detected in Morhulin<sup>®</sup> (38% ZnO), 5% and 50% w/v ZnCl<sub>2</sub> treated skin (Fig. 14c-e). The overall density of zinc observed in the maps of 5% w/v ZnCl<sub>2</sub> and Morhulin<sup>®</sup> (38% ZnO) treated skin appeared similar (Fig. 14c, 14d); the only difference was the concentration of zinc that in the furrow of the latter (Fig. d).

The zinc level detected on the surface of 50% w/v ZnCl<sub>2</sub> treated skin corresponds to the high concentration applied (Fig. 14 e). With the exception of Morhulin<sup>®</sup>, uniform distribution of zinc was observed in the respective element maps of the samples (Fig. 14 a-c, e).





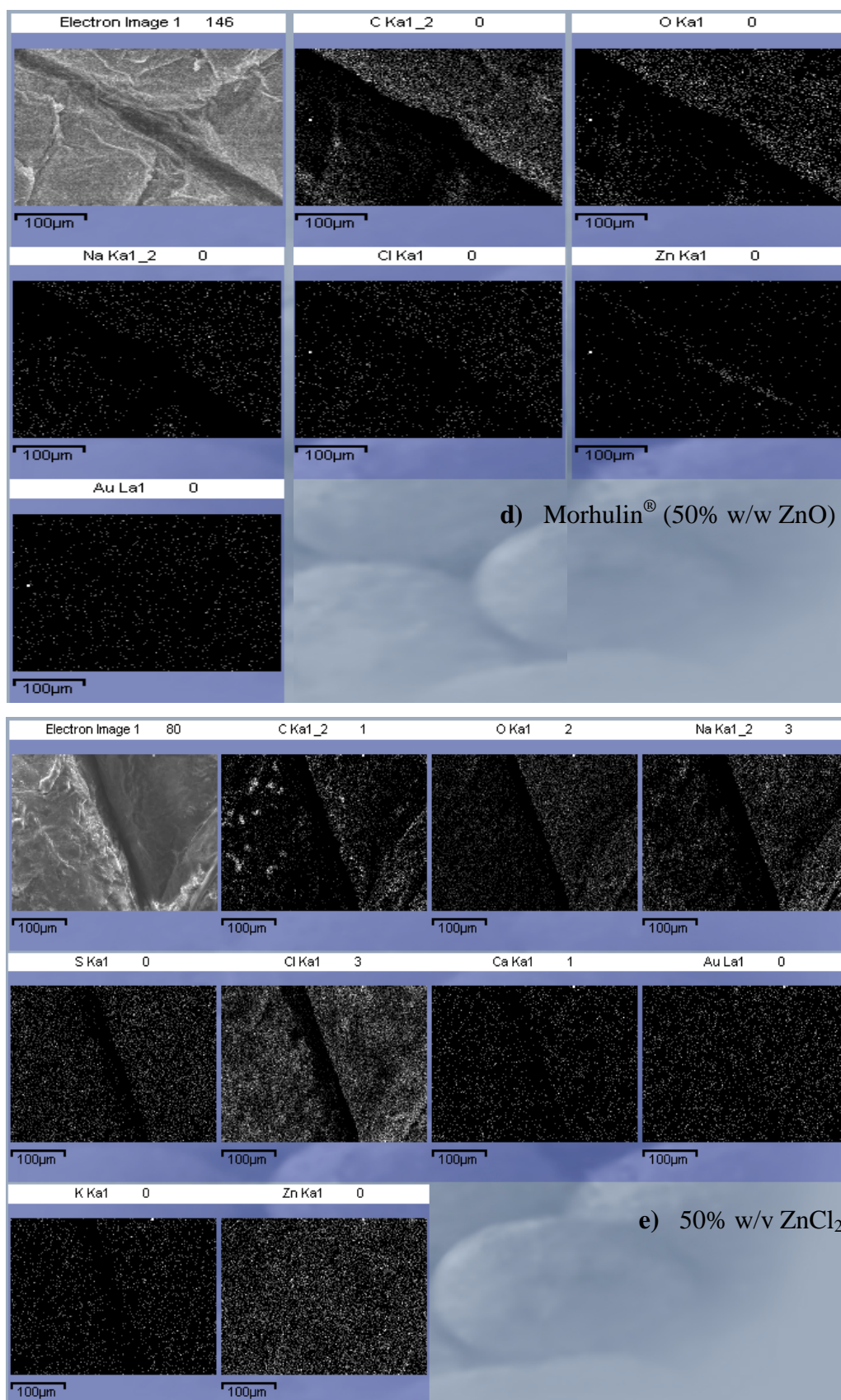


Fig. 14 Element maps showing the distribution of different elements in the proximity of skin furrows following application of a) 2% w/v zinc lactate (20:80, gly:H<sub>2</sub>O), b) 2% w/v zinc lactate (80:20, gly:H<sub>2</sub>O), c) 5% w/v ZnCl<sub>2</sub>, d) Morhulin®, and e) 50% w/v ZnCl<sub>2</sub>



### Smooth areas of skin

No difference was observed in smooth areas of skin after treatment with various zinc preparations (Fig. 15).

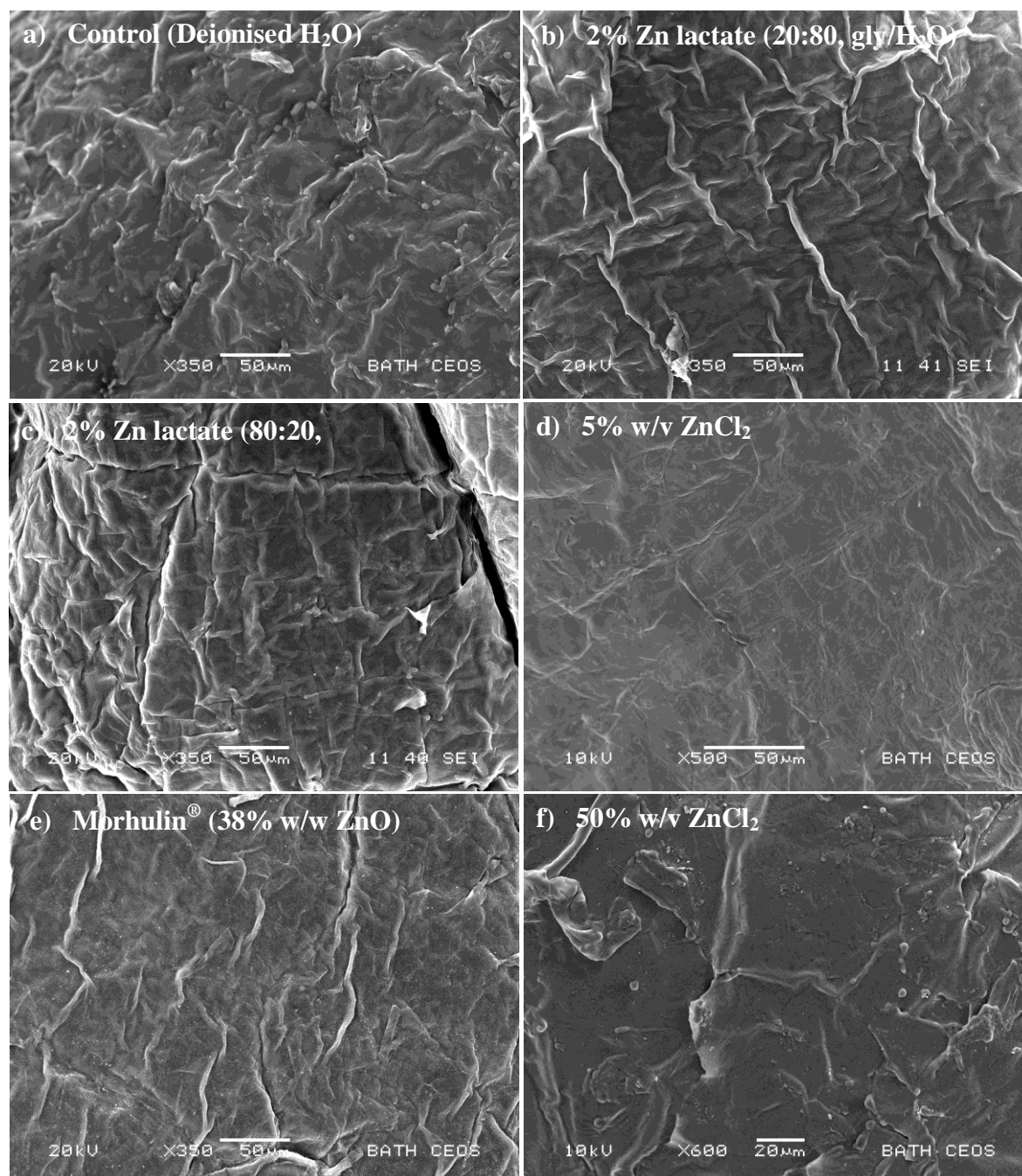


Fig. 15 SEM images of smooth areas of skin following application of a) deionised water (control), b) 2% w/v zinc lactate (20:80, gly:H<sub>2</sub>O), c) 2% w/v zinc lactate (80:20, gly:H<sub>2</sub>O), d) 5% w/v ZnCl<sub>2</sub>, e) Morhulin® (38% w/w ZnO), and f) 50% w/v ZnCl<sub>2</sub>.

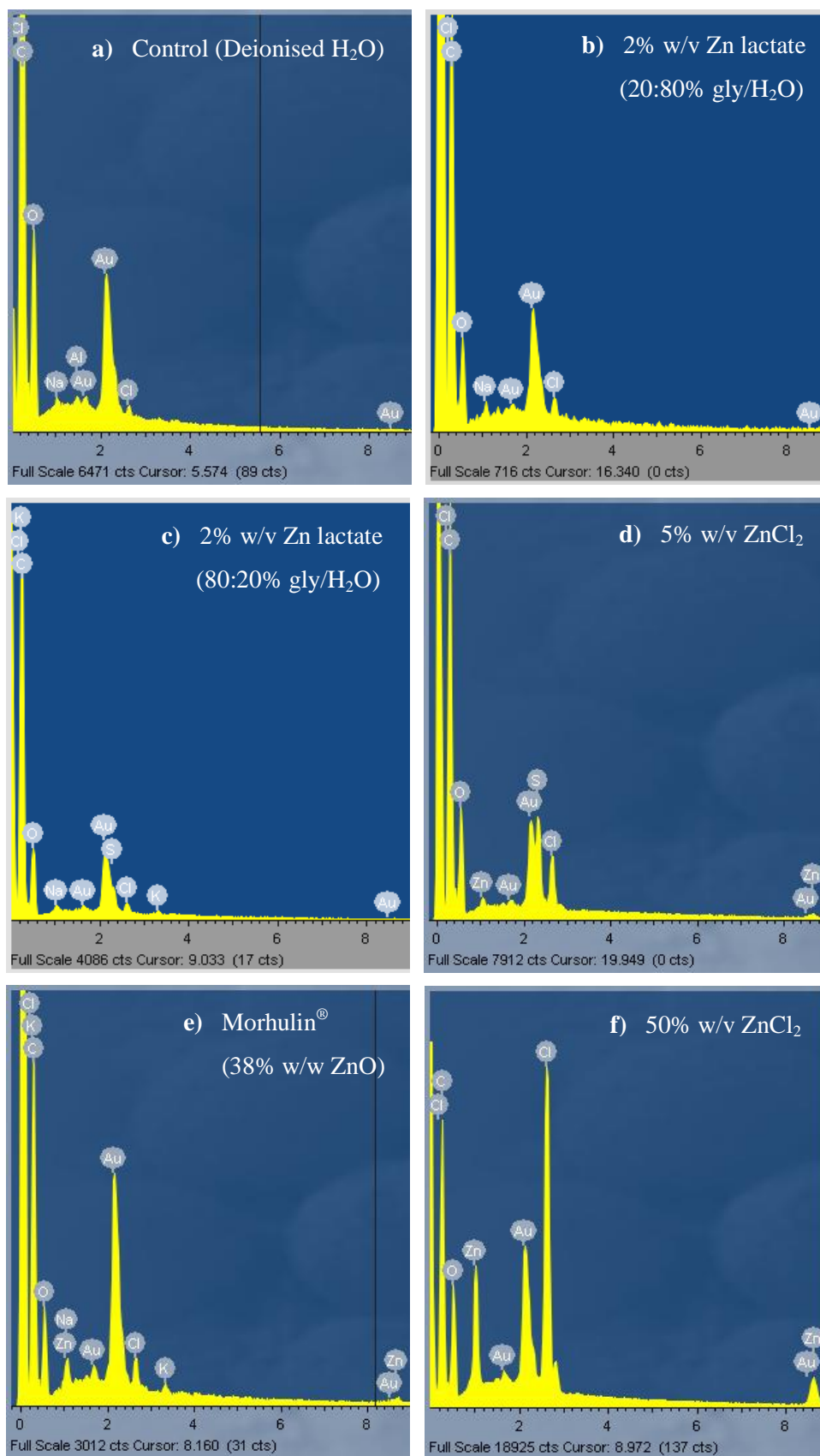
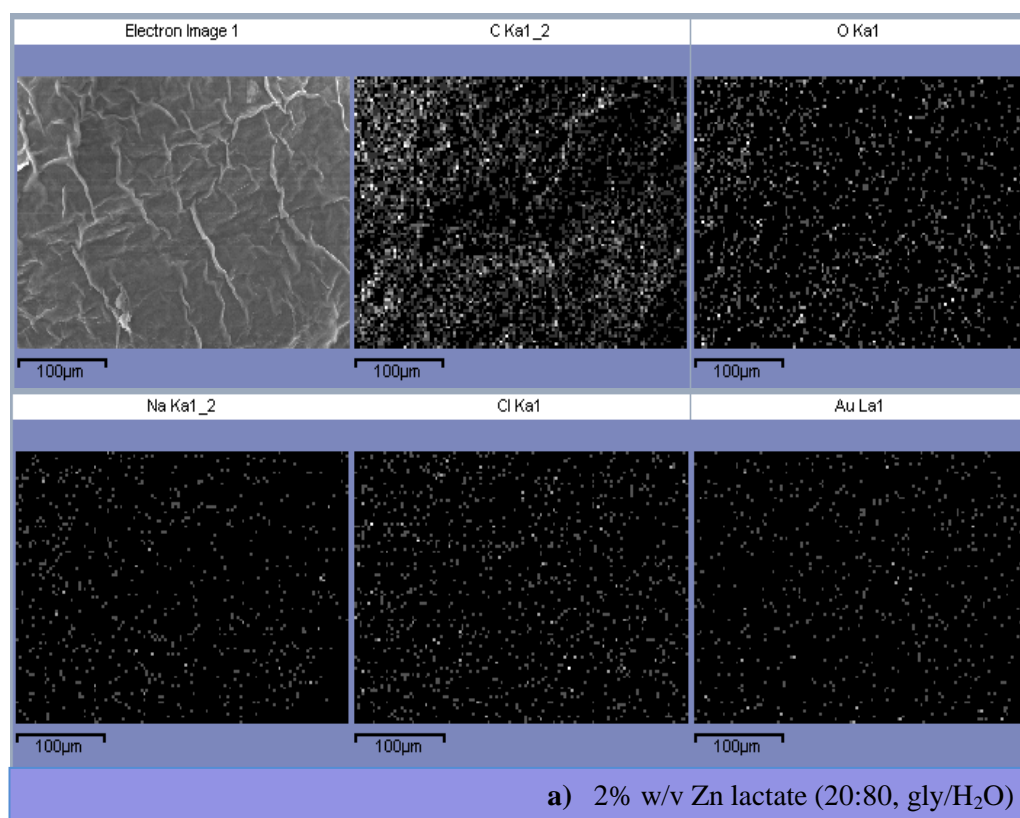
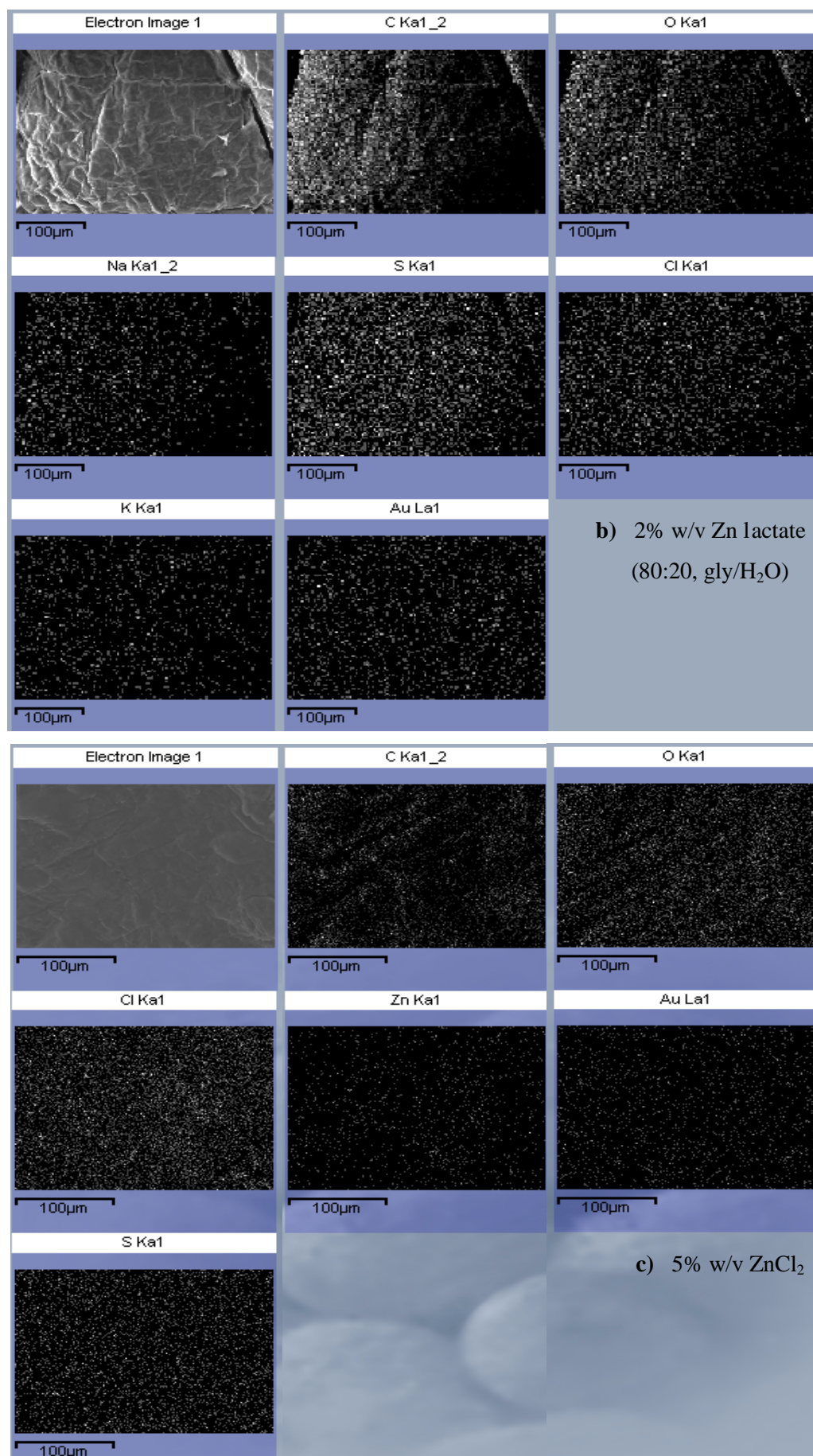


Fig. 16 EDX spectra of smooth areas of skin following application of a) deionised water (control), b) 2% w/v zinc lactate (20:80, gly:H<sub>2</sub>O), c) 2% w/v zinc lactate (80:20, gly:H<sub>2</sub>O), d) 5% w/v ZnCl<sub>2</sub>, e) Morhulin<sup>®</sup> (38% ZnO)<sup>®</sup>, and f) 50% w/v ZnCl<sub>2</sub>.

Fig. 16 shows the EDX spectra of skin samples treated with different concentrations of zinc in a variety of formulations. No zinc was detected in the control (deionised water) or in 2% w/v Zn lactate treated skin (Fig. 16a-c). Small zinc peaks were observed in 5% w/v ZnCl<sub>2</sub> and Morhulin<sup>®</sup> treated skin (Fig. 16d, e). Overlap of the zinc and sodium peaks in Fig. 16e makes comparison difficult with zinc peaks from other formulations. The zinc peak corresponding to 50% w/v ZnCl<sub>2</sub> treated skin was the largest of all (Fig. 16f).

Fig. 17 shows element maps of smooth skin. Zn lactate treated skin did not show any zinc on the surface of the skin (Fig. 17a-b). Amounts and distribution of zinc on skin treated with Morhulin<sup>®</sup> and 5% ZnCl<sub>2</sub> were similar (Fig. 17c-d). 50% w/v ZnCl<sub>2</sub> treated skin showed the highest levels of zinc (Fig. 17e). All elements detected in EDX spectra were observed in the element maps (Fig. 16-17). Zinc levels measured in the EDX spectra (Fig. 16 d-f) appeared to correlate with those visible in the element maps (Fig. 17 c-e).







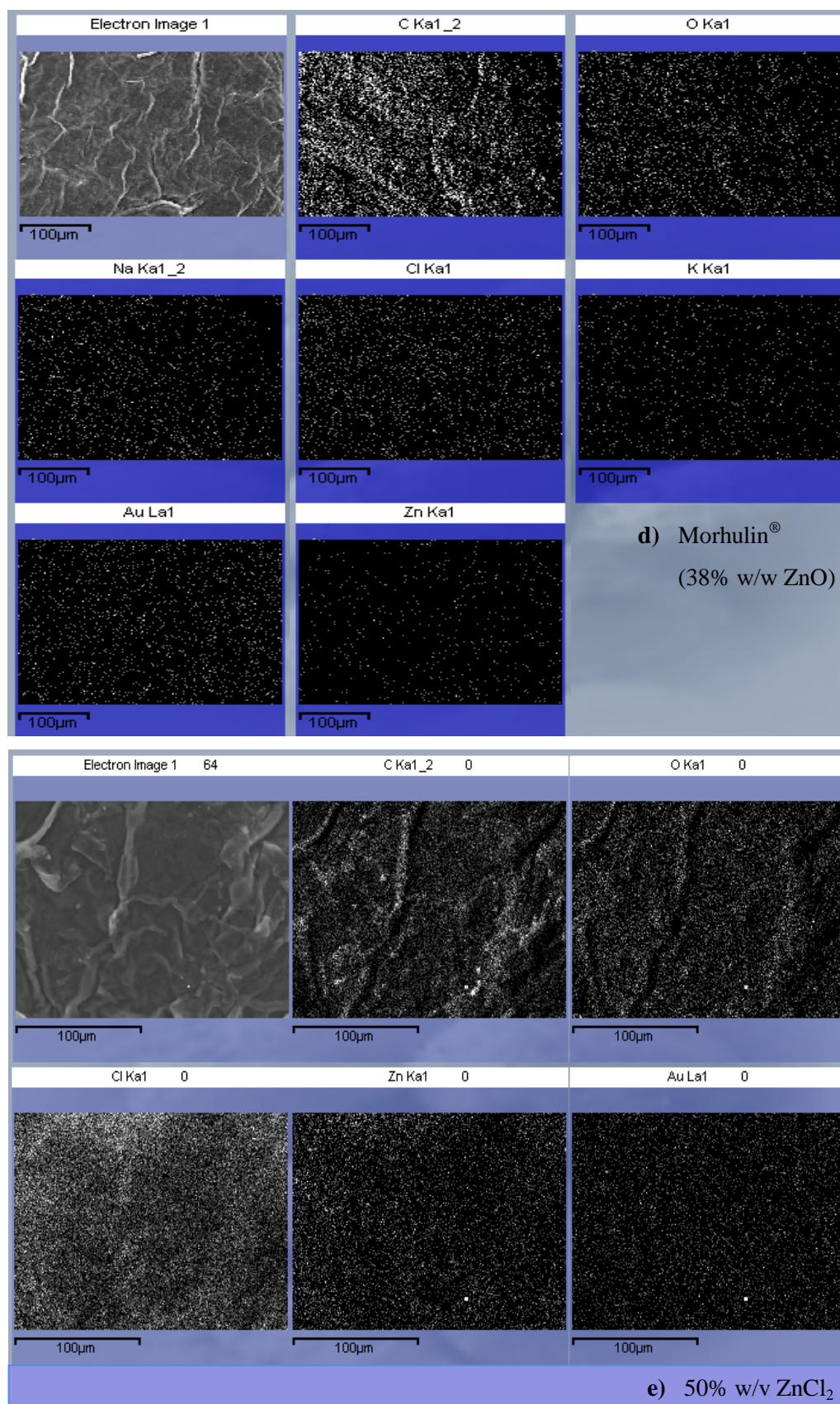


Fig. 17 Element maps showing the distribution of different elements in the proximity of smooth skin following application of a) 2% w/v zinc lactate (20:80, gly:H<sub>2</sub>O), b) 2% w/v zinc lactate (80:20, gly:H<sub>2</sub>O), c) 5% w/v ZnCl<sub>2</sub>, d) Morhulin®, and e) 50% w/v ZnCl<sub>2</sub>

### Rough areas of skin

SEM images of rough skin areas shown in Fig. 18. No differences in appearance after treatment with the various zinc preparations were observed.

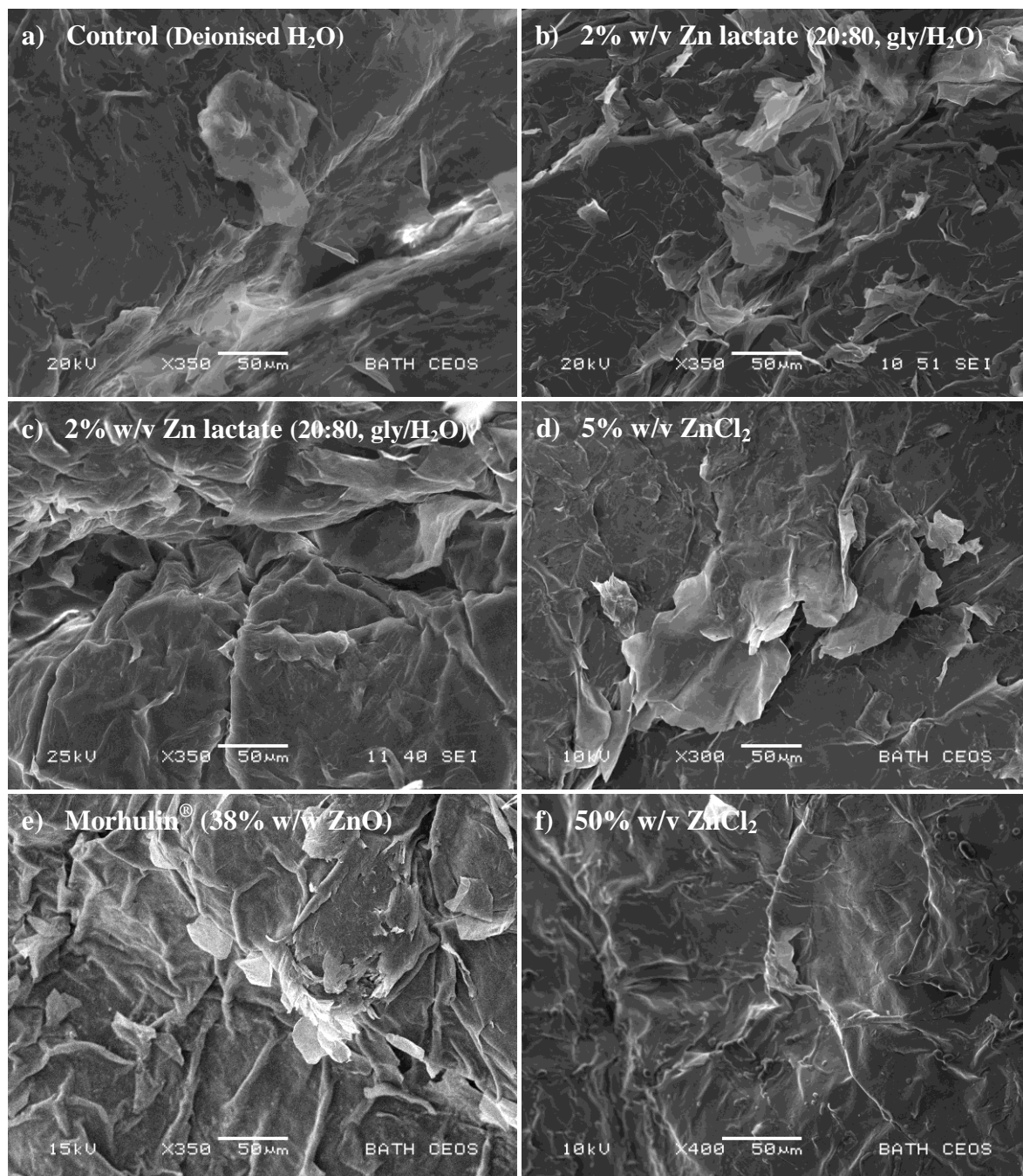


Fig. 18 SEM images of smooth areas of skin following application of a) deionised water (control), b) 2% w/v zinc lactate (20:80, gly/H<sub>2</sub>O), c) 2% w/v zinc lactate (80:20, gly/H<sub>2</sub>O), d) 5% w/v ZnCl<sub>2</sub>, e) Morhulin<sup>®</sup> (38% w/w ZnO), and f) 50% w/v ZnCl<sub>2</sub>.

EDX spectra revealed no zinc on rough skin surfaces of the control and of 2% w/v Zn lactate treated skin (Fig. 19a-c). Skin treated with Morhulin<sup>®</sup> (38% w/w ZnO), and 5% and 50% ZnCl<sub>2</sub> clearly showed the presence of zinc detected with the level following treatment peak of 50% w/v ZnCl<sub>2</sub> being the largest (Fig. 19d-f).

Element maps of rough surfaces are in Fig. 20. No zinc was detected on zinc lactate treated skin (Fig. 20a, b). Similar levels of zinc were detected on skin treated with 5% w/v ZnCl<sub>2</sub> and Morhulin<sup>®</sup> (Fig. 20c-d). The amount of zinc observed on skin treated with 50% w/v ZnCl<sub>2</sub> was highest (Fig. 20e).

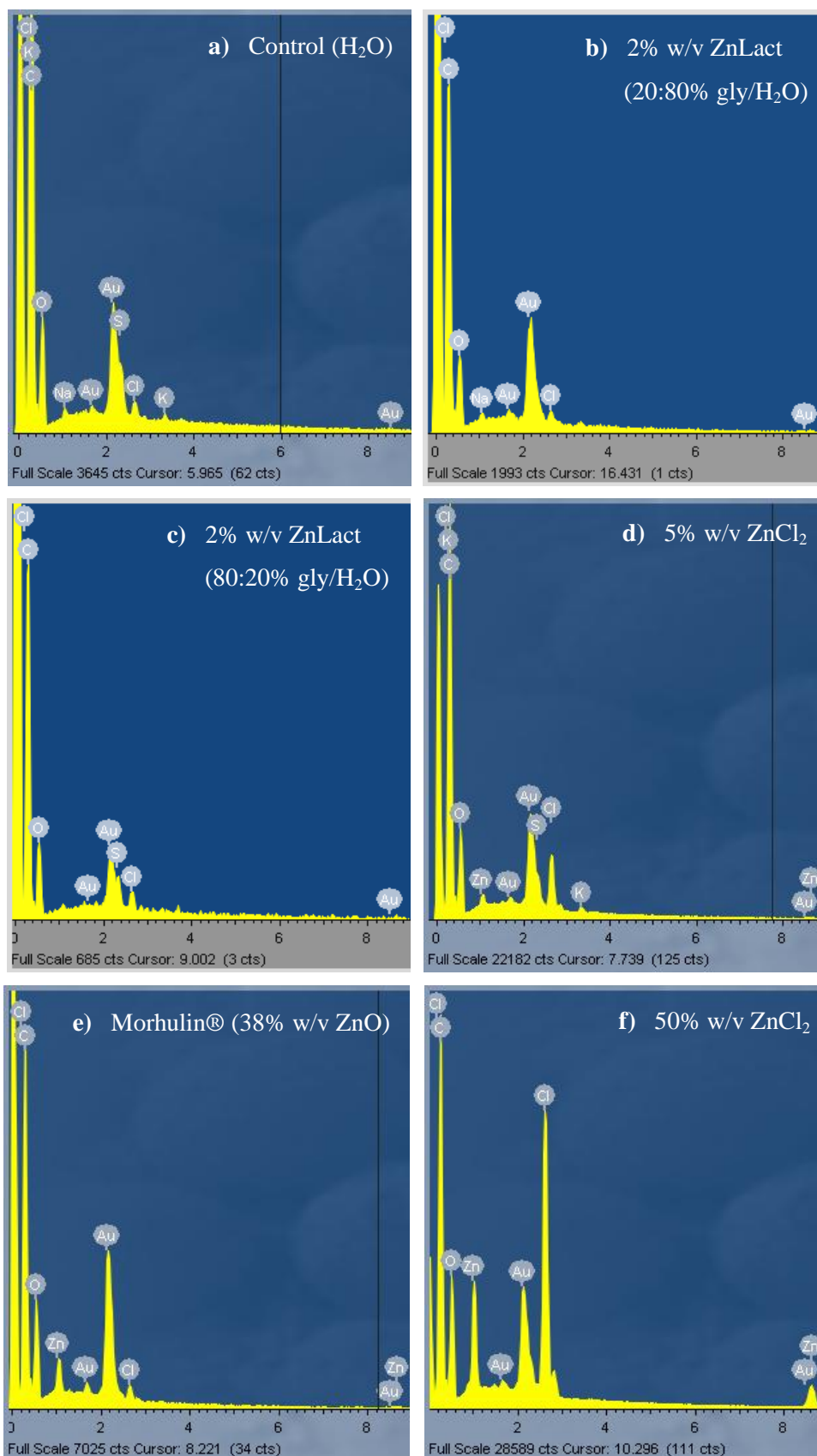
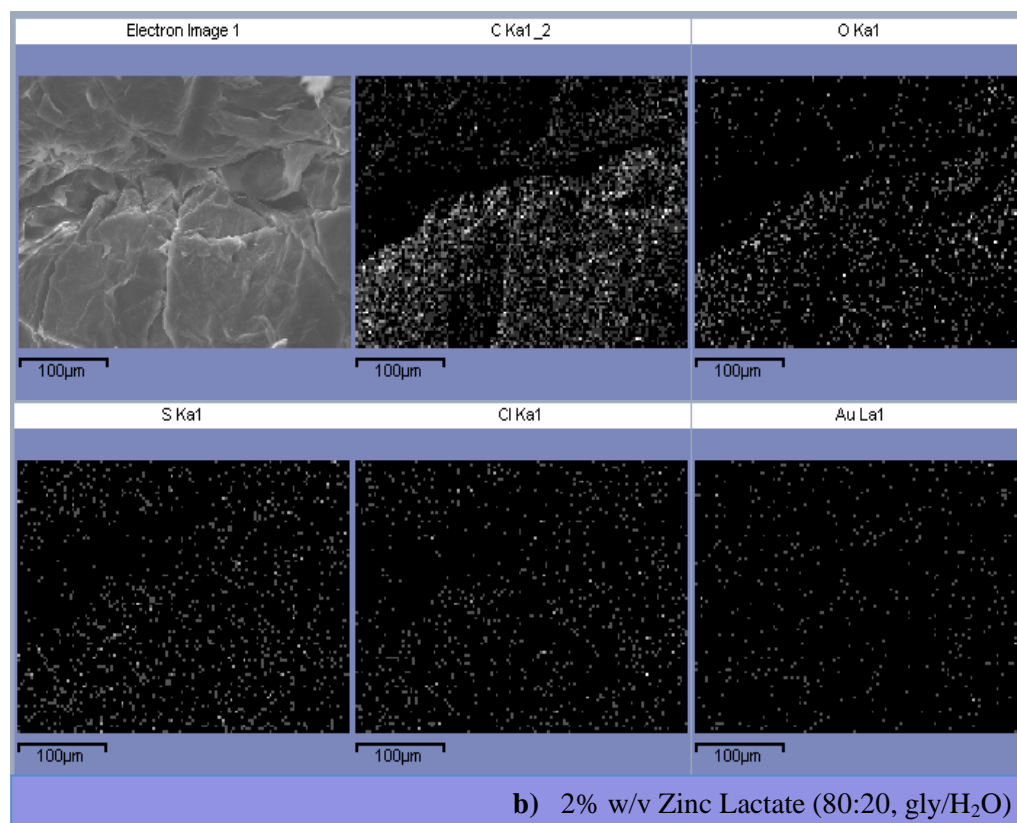
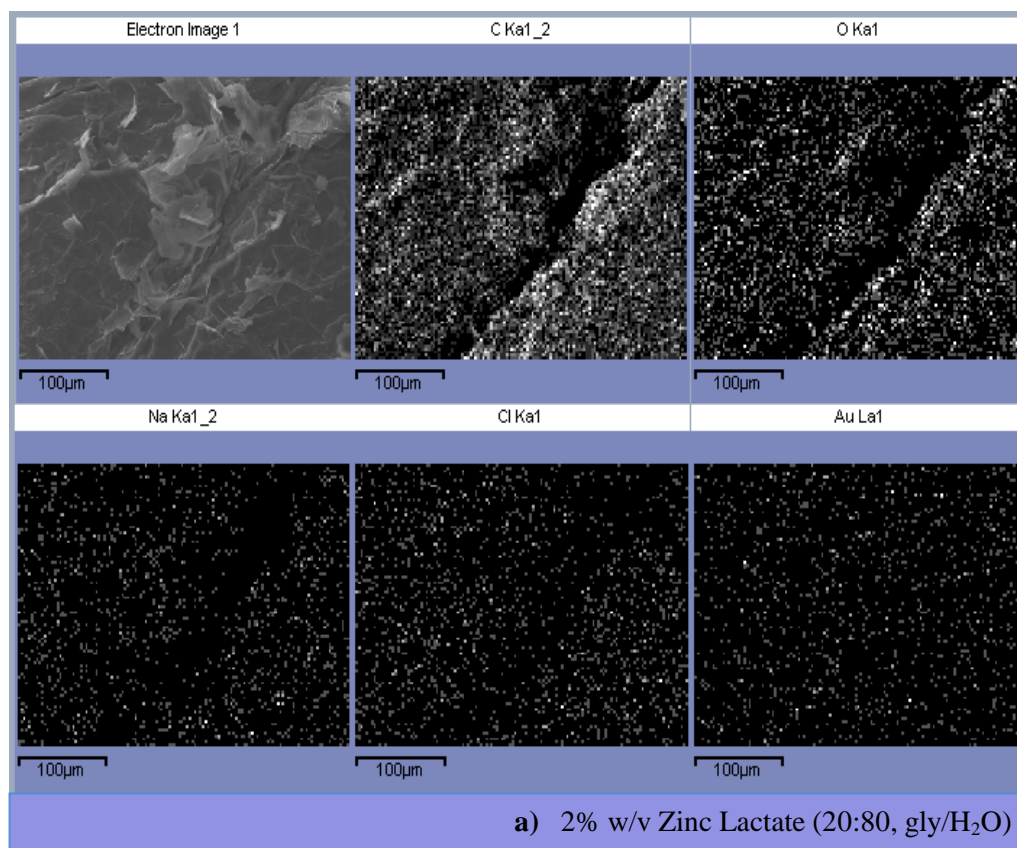
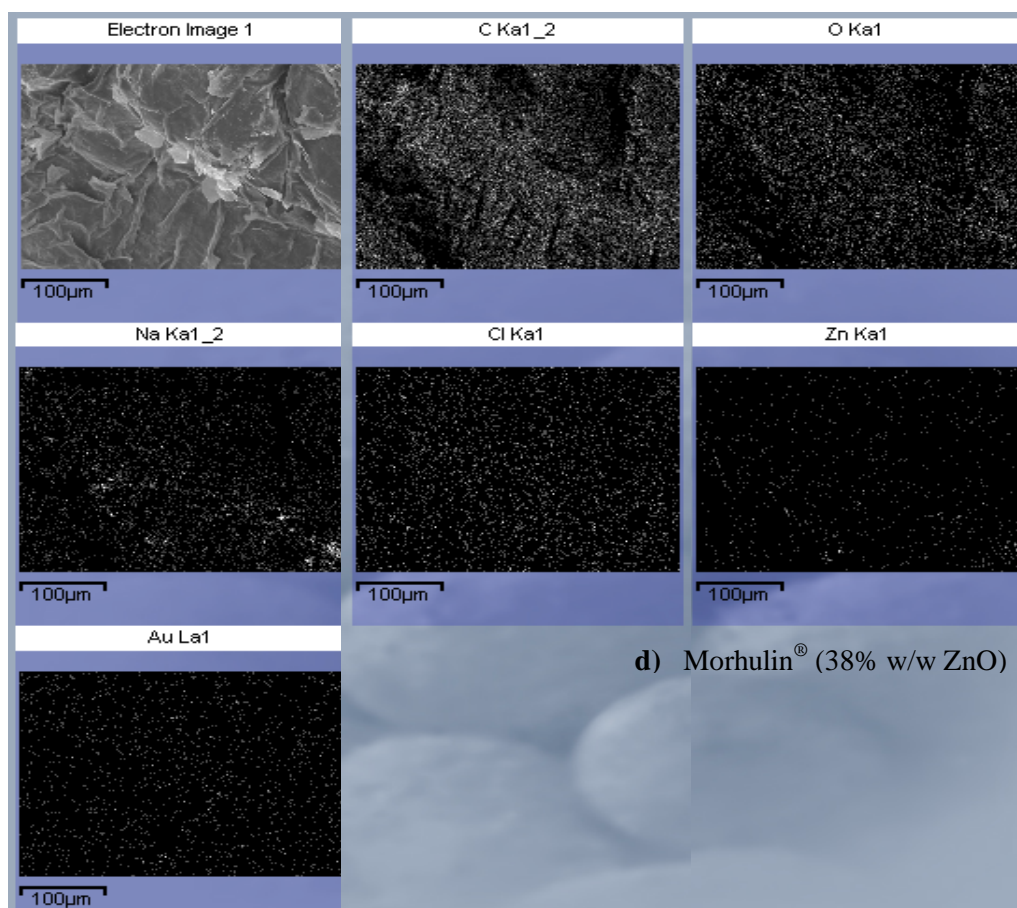
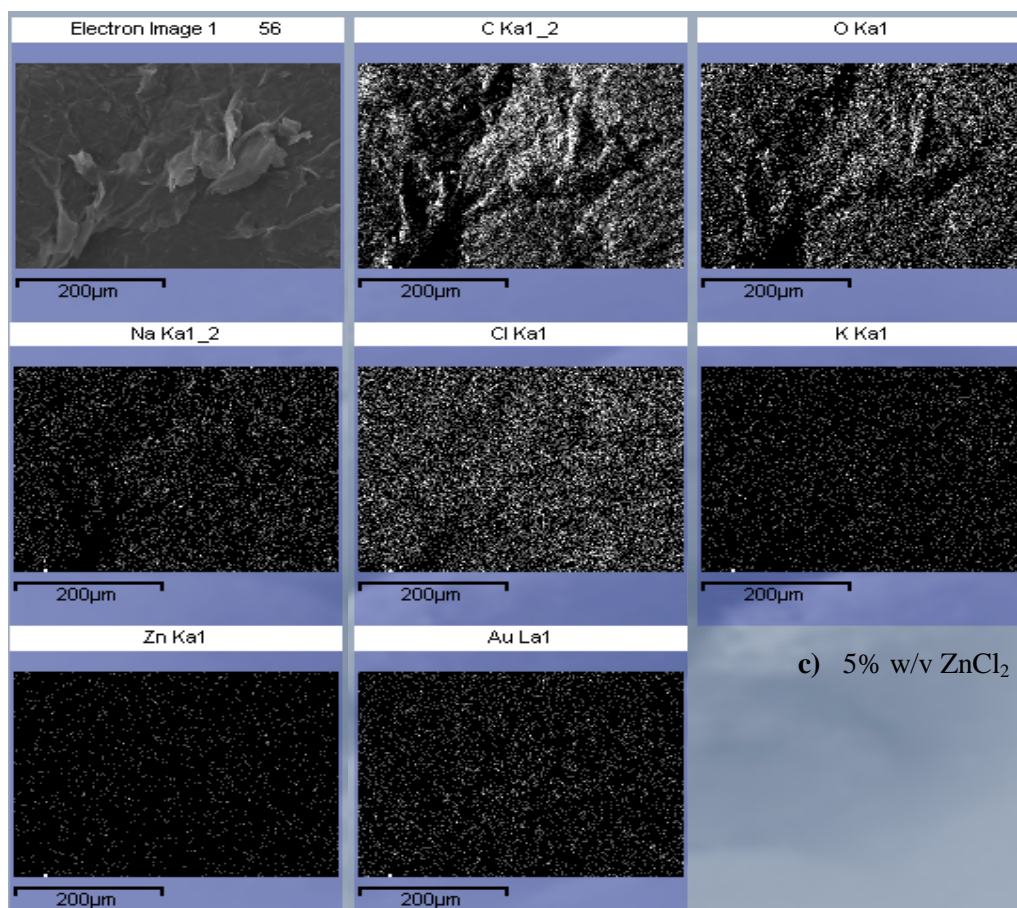


Fig. 19 EDX spectra of rough areas of skin following application of a) deionised water (control), b) 2% w/v zinc lactate (20:80, gly: $\text{H}_2\text{O}$ ), c) 2% w/v zinc lactate (80:20, gly: $\text{H}_2\text{O}$ ), d) 5% w/v  $\text{ZnCl}_2$ , e) Morhulin (38%  $\text{ZnO}$ )<sup>®</sup>, and f) 50% w/v  $\text{ZnCl}_2$ .







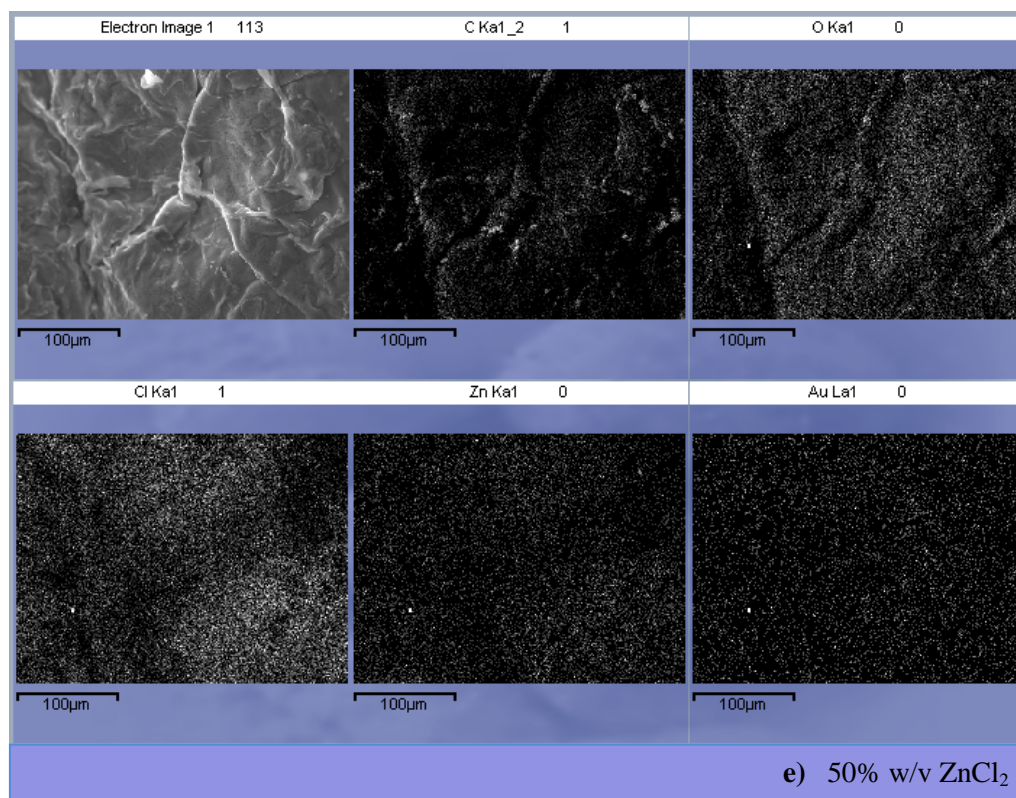


Fig. 20 Element maps showing the distribution of different elements on rough skin following application of a) 2% w/v zinc lactate (20:80, gly:H<sub>2</sub>O), b) 2% w/v zinc lactate (80:20, gly:H<sub>2</sub>O), c) 5% w/v ZnCl<sub>2</sub>, d) Morhulin<sup>®</sup>, and e) 50% w/v ZnCl<sub>2</sub>

## Discussion

The purpose of these experiments was to explore further the delivery of zinc from preparations containing ZnCl<sub>2</sub>, zinc lactate and zinc oxide (Morhulin<sup>®</sup>); changes to the topography of the skin were also examined and the efficiency of two different cleaning procedures (wiping with a dry paper towel and rinsing with deionised water) on zinc distribution and penetration into the skin was assessed. The control experiment considered skin treated with deionised water alone, and evaluated the visual appearance and the presence of naturally occurring elements, including zinc. In these studies, skin was imaged by SEM post-treatment. EDX spectroscopy and elemental mapping was used to determine the distribution, localisation and elemental composition of each skin sample. Due to the heterogeneity of the skin surface, four different structural regions were analysed; hair, furrow, smooth and rough skin. Formulation trapped in these sites makes differentiation between penetration and adsorption of an 'active' (such as zinc) difficult to distinguish.

### Controls

The SEM images (Fig. 1) revealed small, randomly distributed spherical structures on the skin that were entirely organic in composition (results not shown). EDX spectra characterised the elemental composition of the skin surface (Fig.2), and showed the presence of chlorine, carbon, calcium, oxygen, sodium, sulphur (mainly from hair), some aluminium (likely to be contamination) and potassium. Since all samples were coated in gold before analysis, this element was also detected in all EDX and element maps. No naturally-occurring, background zinc was detected in control samples even though previous work (Chapter 3) demonstrated that zinc was present in the stratum corneum at a level of approximately  $1 \mu\text{g}/\text{cm}^2$  ( $\pm 0.6$ ). Presumably, this level was below the limit of detection of EDX spectroscopy and element mapping.

### Distribution of zinc on skin: Comparison between 5% and 50 % w/v $\text{ZnCl}_2$ and between different cleaning methods

Images of the skin after treatment with 5% and 50%  $\text{ZnCl}_2$  showed residue attached to the hair and surrounding areas (Fig. 3a, b). The substance, which was not present on control samples (Fig. 1a), was assumed to be  $\text{ZnCl}_2$  since it was readily removed by rinsing the sample under deionised water indicating that it was water soluble (Fig. 3c-d) (as is  $\text{ZnCl}_2$ ). In element maps of hair on skin treated with 50% w/v  $\text{ZnCl}_2$ , the zinc decreased after rinsing with water was observed (Fig. 5b, d). Rough areas on skin treated with 50% w/v  $\text{ZnCl}_2$  also showed deposits on the surface, which was not present on skin rinsed with water after treatment (Fig. 6f, h). With the exception of the aforementioned areas, SEM images of furrows, smooth areas treated with 5% and 50% w/v  $\text{ZnCl}_2$ , and also rough areas on skin treated with 5% w/v  $\text{ZnCl}_2$ , showed no differences in topography between zinc treatments or cleaning procedures was observed (Figs. 3c-h, 6a-e, g).

The presence of zinc on the surface of the skin is shown in the EDX spectra (Fig.4). While it was not possible to quantify the amounts of zinc detected however, relative amounts were estimated by comparing peak heights and size. EDX spectra showed that 50% w/v  $\text{ZnCl}_2$  delivered higher levels of zinc to all skin areas than 5% w/v  $\text{ZnCl}_2$ . These results were supported by corresponding element maps. EDX spectra also revealed that the levels of zinc on the skin were affected by cleaning methods, i.e., rinsing with deionised water removed much of the zinc adhered to the skin surface. However, element maps did not completely overlap with these results; possibly due to its lower sensitivity than EDX spectroscopy.(14)

### Comparison of zinc delivery from different formulations

Five different zinc preparations, (i) 2% w/v zinc lactate (20:80, gly:H<sub>2</sub>O), (ii) 2% w/v zinc lactate (80:20, gly:H<sub>2</sub>O), (iii) 5% w/v ZnCl<sub>2</sub>, (iv) Morhulin<sup>®</sup> (38% w/w ZnO), (v) 50% w/v ZnCl<sub>2</sub> were considered. Deionised water was used for the control. With the exception of Morhulin<sup>®</sup>, there were no differences in terms of skin appearance between post-treatment (Fig. 9, 12, 15, 18). SEM images of Morhulin<sup>®</sup> (38% w/w ZnO) treated skin showed a thin, opaque/white coating over all four areas analysed (Fig. 9e, 12e, 15e, 18e). This was largely due to the difficulty in removing the (viscous and oily) formulation from the skin even when isopropyl alcohol wipes were used.

When skin was treated with 2% zinc lactate, with the exception of very low levels in skin furrows (Fig. 13b), EDX showed no zinc in any other areas (Fig. 10, 16, 19 a-c, and 13a, c). Small amounts of zinc were found in all four areas of skin treated with 5% w/v ZnCl<sub>2</sub> (Fig. 10, 13, 16, 19c). EDX spectra of hairs showed similar levels of zinc after application of Morhulin<sup>®</sup> and 50% w/v ZnCl<sub>2</sub>. However, element maps indicated that zinc delivery from Morhulin<sup>®</sup> was less than that from 50% w/v ZnCl<sub>2</sub>. Zinc levels in skin furrows, and on smooth and rough areas of Morhulin<sup>®</sup> treated skin were small compared to those after application of 50% w/v ZnCl<sub>2</sub>. Element maps showed similar levels of zinc after treatment with either 5% w/v ZnCl<sub>2</sub> or Morhulin<sup>®</sup>; for the latter, zinc accumulation was observed in skin furrows (Fig. 14d). This discovery was not surprising since the surface of the skin is not homogeneous and provides several places in which the formulation may become trapped and from which it is difficult to remove (even with isopropyl alcohol wipes). This finding is consistent with an earlier study of the dermatopharmacokinetics of betamethasone 17-valerate that also revealed sequestration of the formulation in skin furrows.(15)

Background levels of chlorine (thought to be naturally occurring) were also observed in each EDX spectrum with ZnCl<sub>2</sub> treated skin showing higher levels than others. Element maps also displayed high levels of chlorine after application of ZnCl<sub>2</sub> with quantities in rough proportion to the concentration applied.

In summary, the experiments revealed that (i) 50% w/v ZnCl<sub>2</sub> delivered zinc more effectively to the skin than the 5% solution, (ii) rinsing the skin with water post-treatment removed a large fraction of the zinc adsorbed/penetrated into the SC, (iii) the zinc lactate preparation was not successful in delivering zinc to the skin.

SEM imaging, EDX microanalysis and element mapping offers useful tools with which to examine the surface of the skin and provide information regarding the distribution and relative levels of zinc delivered from different formulations. However, the use of EDX and element mapping are limited by sensitivity, and the acquisition of even semi-quantitative data requires that specific measurement criterion are met; for example, each sample must be completely flat and exactly the same distance from the microscope lens. This cannot be achieved without prior arduous and complicated sample preparation.



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**Chapter Seven: Absorption of Zinc from Sudocrem<sup>®</sup> into Human  
Stratum Corneum *In Vivo*: Comparison of Uptake Determined  
by Different Extraction Methods**



## **Chapter Seven**

### **Absorption of zinc from Sudocrem<sup>®</sup> into human stratum corneum *in vivo*: comparison of uptake determined by different extraction methods**

#### **Summary**

*Background* - Currently, there is a multitude of zinc containing products available on the market. These range from barrier products for contact dermatitis/nappy rash, through medicated bandages for eczema and psoriasis, to sunscreens to reflect harmful UV-rays. However, the percutaneous penetration of zinc oxide from these is limited (and, in the case of sunscreens, undesirable) as their main function is to provide a mechanical barrier to protect the skin from assault due to irritants. The development of a novel topically applied zinc-containing formulation for reparation of the defective skin barrier that occurs in eczema is in progress. Therefore, it would be pertinent to investigate the percutaneous penetration of zinc from one of the most popular products; Sudocrem<sup>®</sup>

*Objectives* - To determine the penetration of zinc into human stratum corneum *in vivo* following application of Sudocrem<sup>®</sup> (15.25% w/w ZnO) once (x1) to three times (x3) a day, and subsequently to compare tape stripping (TS), reverse iontophoresis (RI) and passive extraction (PE) for quantitation of zinc uptake into the skin.

*Methods* - Sudocrem<sup>®</sup> (15.25% w/w ZnO) was applied to the forearm of eight healthy volunteers for two weeks. Each arm was 'divided' into control and treated regions. One arm was treated with the cream once a day, the other three times. After the treatment period, the zinc was extracted from the skin by tape stripping (TS), reverse iontophoresis (RI) and passive extraction (PE). Levels of zinc were analysed by inductively coupled plasma - atomic emission spectroscopy (ICP-AES).

*Results* - Significant statistical differences in mean cumulative concentrations of zinc extracted by tape stripping and passive extraction were detected between treatments (control, once and three times Sudocrem<sup>®</sup> application). No significant difference in mean cumulative concentrations of zinc extracted by cathodal and anodal RI were detected. Significant statistical differences was also detected between extraction methods for once and three-times daily applications.

*Conclusions* - Tape stripping recovered the most zinc from the skin. Zinc sequestered in skin furrows contributed to that which was extracted from the stratum corneum thereby

resulting in higher concentrations measured. A statistical difference was detected in zinc uptake into the stratum corneum between once and three times daily applications of Sudocrem<sup>®</sup>.

Keywords: Sudocrem<sup>®</sup>, tape stripping, reverse iontophoresis, passive extraction, percutaneous penetration, zinc oxide

## **Introduction**

Zinc-containing products are widely available on the market, many of which are barrier preparations such as sunscreens or treatments for nappy rash.<sup>(1)</sup> Their purpose is to provide a protective mechanical barrier that remains on the skin.<sup>(1)</sup> Penetration of zinc from such formulations is largely limited to the upper layers of the stratum corneum.<sup>(2-7)</sup> A detailed report published by the European Union's Scientific Committee on Cosmetic Products and Non-Food Products (SCCPNFP) concluded that no change in zinc plasma levels occurred *in vivo* in human subjects treated with a daily application of 40% zinc oxide ointment for 10 days; penetration of zinc was less than 1% of the applied dose and the majority of zinc oxide was recovered from the outermost layer of the skin, the stratum corneum.<sup>(8)</sup>

However, with respect to formulating a novel treatment for eczema, it is desirable for zinc to be able to penetrate into the stratum corneum to elicit its therapeutic effects. The weakened skin barrier in eczema is xerotic and prone to forming cracks that facilitate the percutaneous influx of xenobiotics, microorganisms and allergens.<sup>(9-13)</sup> As a consequence, the sufferer experiences itching and soreness in the affected areas, which leads to scratching and the eventual formation of lesions, and in some cases, bleeding.<sup>(10, 11, 14-17)</sup> These lesions take weeks to heal and provide additional and more direct pathways through the skin for irritants and allergens to cause further damage and inflammation, and thus delaying the healing process. The inadequate protection of the skin barrier is a direct result of the lack of natural moisturising factor and the increased desquamation of the stratum corneum. Natural moisturising factor keeps the skin hydrated, helps maintain flexibility and provides resistance to tearing.<sup>(18)</sup> Increased desquamation reduces the thickness of the skin barrier causing it to be fragile and more prone to the development of cracks.<sup>(18)</sup> Further, the thin barrier facilitates the ingress of irritants and allergens across the stratum corneum to deeper skin layers where they can cause irritation and discomfort. Penetration of zinc from topically applied formulations may aid barrier repair. In formulating a novel treatment for such purposes, it is pertinent to examine the performance of a commercially available zinc-containing product in terms of the penetration of zinc into the skin.

This report details the absorption of zinc into the skin of human forearms *in vivo* from a well-known and highly-used commercial nappy rash formulation, Sudocrem®.

Determination of the most efficient method for the extraction and quantitation of zinc from the skin has also been investigated.

## **Materials and Methods**

### **Materials**

Sudocrem<sup>®</sup> (15.25% zinc oxide) was purchased from Forest Tosara, Dublin, Ireland, Scotch No. 845 book tape was purchased from 3M (St. Paul, MN, USA), Micropore<sup>™</sup> tape was from 3M (Nadarzyn, Poland). The tape stripping template (2 cm diameter aperture) was constructed from book tape. Silver wire (> 99.99% purity) and silver chloride (> 99.999%) were purchased from Sigma-Aldrich Co. (Gillingham, UK). Glacial acetic acid (HLPC grade) was from Fisher Scientific. The reverse iontophoresis extraction solution comprised of 3 mM saline. All solutions were prepared with deionised water, 18.2 MΩ-cm (NANOpure<sup>®</sup> DIamond<sup>™</sup> Life Science (UV/UF) Ultrapure Water System, Barnstead International, Dubuque, Iowa, USA). Sodium chloride was purchased from Acros Organics (Belgium).

### **Study Population**

Six female volunteers, aged 19-55 years, participated in the study. All were healthy, had no history of skin disease, and did not suffer from any dermatological conditions at the time of the study. An information pack containing Volunteer Information Sheets, Notes for Volunteers, consent form and a cream application chart was given to each participant. The documents contained the project details and additional information about the cream. Any questions raised were answered appropriately and written informed consent was obtained from each participant. Participants were asked to refrain from applying any topical formulations to the test areas, i.e., their forearms, throughout the duration of the study and to avoid taking part in any activities that may affect the properties of the skin such as sunbathing, swimming, exfoliation and beauty treatments. They were also instructed to allow at least 2-3 hours post-application before engaging in activities that may change the environment of the treatment site; e.g., taking a bath/shower or sporting activities. The study was approved by the Bath Research Ethics Committee (Reference: 09/H0101/36).

### Treatment with a Zinc-containing Formulation

Volunteers were asked to apply Sudocrem<sup>®</sup>, a commercially available zinc-containing formulation, onto their ventral forearms for a period two weeks. The ventral forearm was divided into two regions, treated and untreated, by an imaginary line drawn from wrist to elbow. One arm was designated for cream application once a day, the other arm for three times a day. On the treated sides of each arm, 2ml of the zinc formulation was measured and applied using a (needleless) syringe either once or three times a day for two weeks. The cream was massaged onto the skin until a uniform layer was achieved. After ten minutes the excess cream was removed by gently wiping with a soft facial tissue (Fig. 1). Care was taken to avoid spreading the cream onto the untreated side.

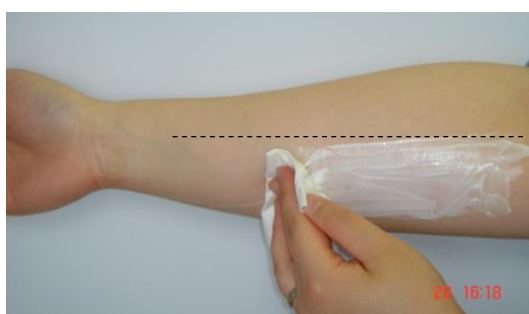


Fig. 1 The ventral forearm divided into treated and untreated sides. Sudocrem<sup>®</sup> was applied, and after 10 minutes, was carefully removed without contaminating the contralateral (control) side of the forearm

When the cream was applied three times a day, treatments were conducted in the morning, afternoon and night. After applying the cream for two weeks the volunteers were subjected to a series of zinc extraction experiments. Cream application was discontinued 24 hours prior to these procedures.

### Zinc Extraction Methods

#### 1. Tape Stripping

Prior to commencement of tape stripping, volunteers acclimatised to the laboratory environment for 20 minutes and the entire forearm was cleaned with an isopropyl alcohol wipe and allowed to dry before starting the procedure. A template with an aperture of approximately 2 cm diameter was firmly secured over the sampling site to delimit the area. Baseline transepidermal water loss (TEWL) was measured (Biox Aquaflux AF102, Biox



Systems Ltd. London, UK). A pre-weighed tape-strip (Sartorius AG SE-2F semi-microbalance, Sartorius AG, Germany) was placed onto the sampling site and pressed firmly to ensure good contact between the two surfaces. The tape was then removed with one swift movement and TEWL re-measured. Alternate tape stripping and TEWL measurements were made until a value of approximately four times the baseline was achieved. The tapes were then re-weighed and the zinc subsequently extracted from them. The following day, the tapes were re-weighed, rolled and placed into 4 ml sample vials with 1.5 ml 10% acetic acid solution to extract the zinc. The vials were shaken overnight using an IKA HS 260 Basic shaker (IKA<sup>®</sup> Werke GmbH & Co., KG, Germany) then stored before analysis.

## 2. Reverse Iontophoresis

Skin sites adjacent to those used for tape stripping were used for reverse iontophoresis, cleaned with isopropyl alcohol wipes and allowed to dry completely prior to starting the procedure. The reverse iontophoretic system consisted of two glass cylinders (volume = 5 ml, surface area approximately 2 cm<sup>2</sup>) in which the anode and cathode were positioned. The chambers were firmly attached to the ventral forearm using medical adhesive tape and silicon grease (Fig. 2). The vials were fitted with caps through which the silver-silver chloride (Ag/AgCl) electrodes were introduced. The two chambers were positioned about 6 cm apart.

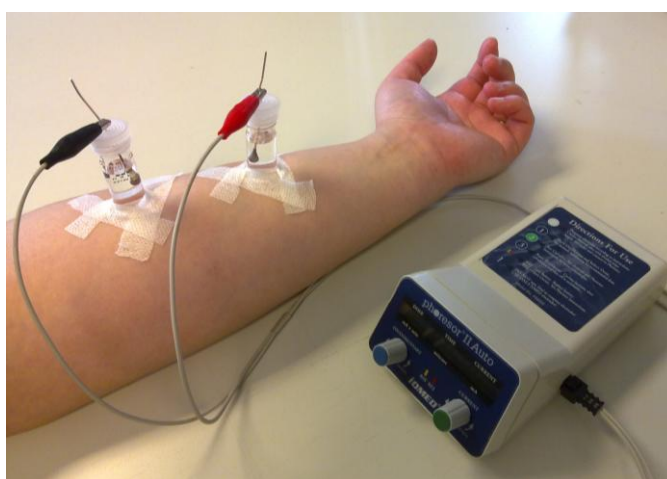


Fig. 2 The reverse iontophoresis set-up

Each chamber was filled with 3 ml of 3 mM saline solution. Current ( $0.3 \text{ mA/cm}^2$ ), was supplied from a commercially-available, U.S. Food and Drug Administration-approved device, the Phoresor II Auto (Iomed Inc., UT, USA) which also has the CE mark. After 15 minutes of iontophoresis, the current was interrupted and the contents of the electrode chambers removed and stored. The chambers were refilled with fresh solution, and reverse iontophoresis was continued for a further 15 minutes to generate a second set of extraction samples. This procedure was carried out on both arms at both treated and untreated sides.

### 3. Passive Extraction

Passive extraction followed an identical procedure as that just described but did not, of course, involve the use of electrodes nor the application of current.

### Sample Analysis

All samples were appropriately diluted with deionised water to ensure that the zinc concentration fell within the range of the analytical instrument. The samples were analysed at the University of Bristol, Department of Earth Sciences using a JY Horiba Ultima inductively coupled plasma sequential atomic emission spectrometer (ICP-AES) (JY Horiba, Japan) fitted with a Burgener T2100 Nebulizer and JY Horiba AS421 autosampler set at a wavelength of 213.856 nm. Calibration standards were prepared from dilution of 1000 ppm Zinc Standard for ICP from Primar F (Fisher Scientific UK Ltd., Loughborough, UK).

### Data Analysis

SC thicknesses were calculated from the TEWL measurements recorded during tape stripping using the approach described in Chapter 2.

### Statistical Analysis

Normality tests, non-linear regression and statistical analyses were performed using GraphPad Prism<sup>®</sup> 4.00 software (Graph Pad Software Inc., San Diego, CA, USA). Statistical significance between data sets was determined via paired two-tailed t-test and one-way ANOVA where appropriate. The confidence interval was adjusted to 95% and the significance level set at  $P < 0.05$ . Outliers, determined by Grubb's test (<http://www.graphpad.com/quickcalcs/index.cfm>), were removed from data analysis.

## Results

### Sudocrem<sup>®</sup> application frequency comparison

The effect of application frequency on the amount of zinc delivered to the skin was investigated.

#### 1. Tape stripping

Stratum corneum thicknesses on the forearm of the six volunteers' ranged from 4.6 - 17.1  $\mu\text{m}$  with an average ( $\pm\text{SD}$ ) thickness of  $11.0 \pm 3.6 \mu\text{m}$ .

The amounts of zinc extracted from the tape strips as a function of depth (x) within the stratum corneum are shown in Fig. 3. Controls (untreated skin) contain the least amounts of zinc (i.e., endogenous levels) with the upper layers of the stratum corneum containing more than those deeper into the membrane.

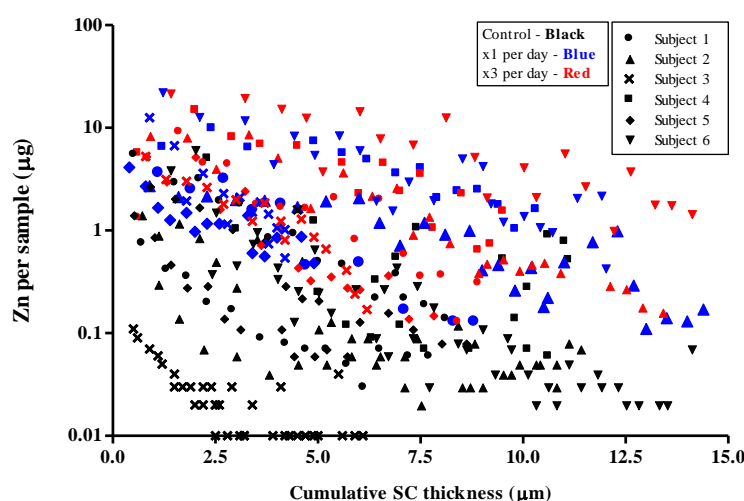


Fig. 3 Profiles of zinc as a function of position within the stratum corneum. Black, blue and red symbols represent control, once and three times a day Sudocrem<sup>®</sup> application, respectively.

Normalisation of stratum corneum thickness allows inter- and intra-variation in zinc content of each volunteer to be examined and compared (Fig. 4) and quite clearly separates the control data from those of the treated skin. However, since the amount of zinc in each sample is dependent on the mass of stratum corneum removed, (and this varies from tape to tape), the data need to be further normalised before the most appropriate comparisons can be made.

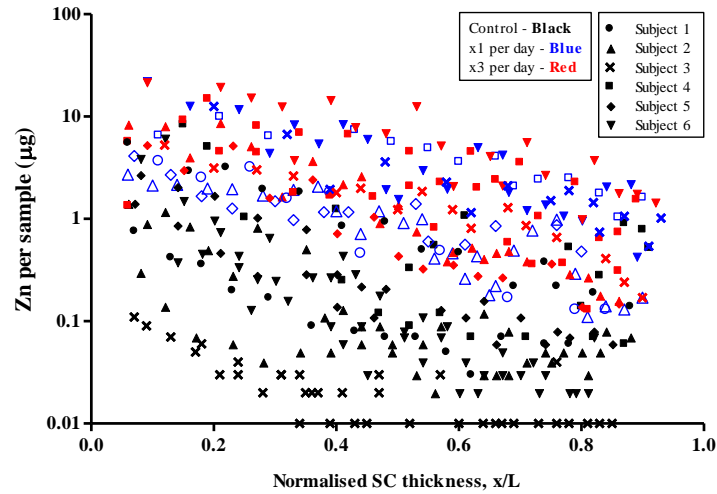


Fig. 4 Amounts of zinc per sample as a function of normalised stratum corneum thickness

Calculating the amounts of zinc per unit mass of stratum corneum standardises the amount of zinc detected at different depths of the stratum corneum. Though this manipulation further separates the control group from the treatment groups, no segregation between once and three times a day treatments is obvious (Fig. 5)

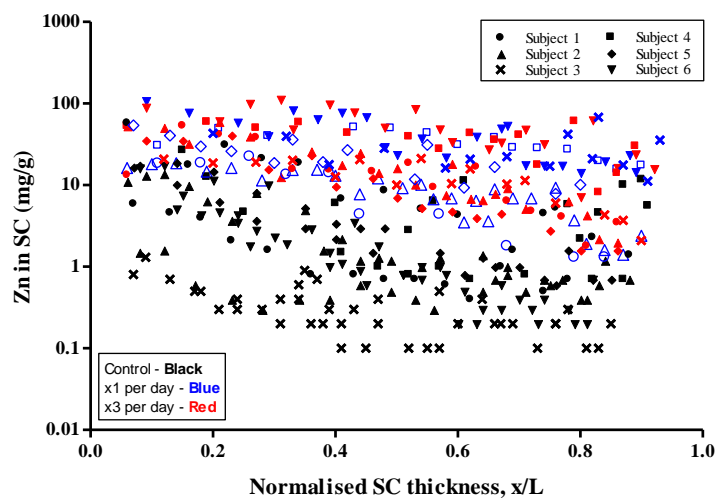


Fig. 5 Further normalisation of the amounts of zinc (quantity per unit mass of stratum corneum) as a function of normalised stratum corneum depth

The cumulative amounts of zinc in the stratum corneum were also determined and are plotted as a function of stratum corneum position in Fig. 6. Though the control group is well separated from the treatment groups, overlap of the data from the latter is still evident.

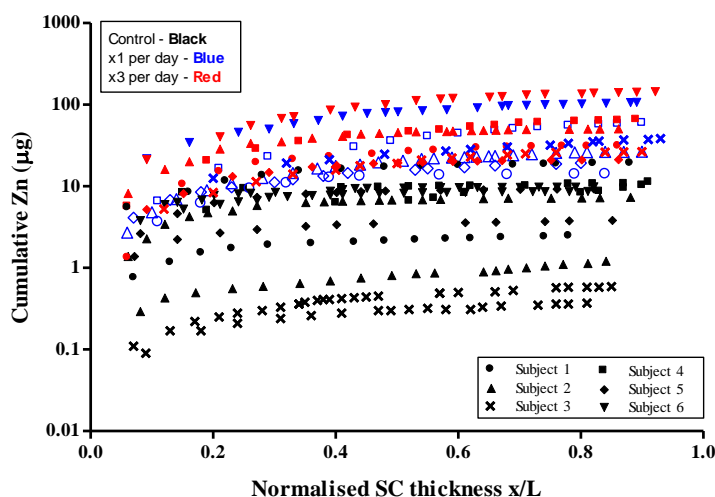


Fig. 6 Cumulative zinc extracted from tape strips as a function of position in the stratum corneum

A statistical analysis of cumulative zinc uptake into the stratum corneum following once, or three times a day treated with Sudocrem® was undertaken and a two-tailed paired t-test revealed a significant difference ( $P = 0.03$ ) between both treatments despite the evident variability in the results (Fig. 7).

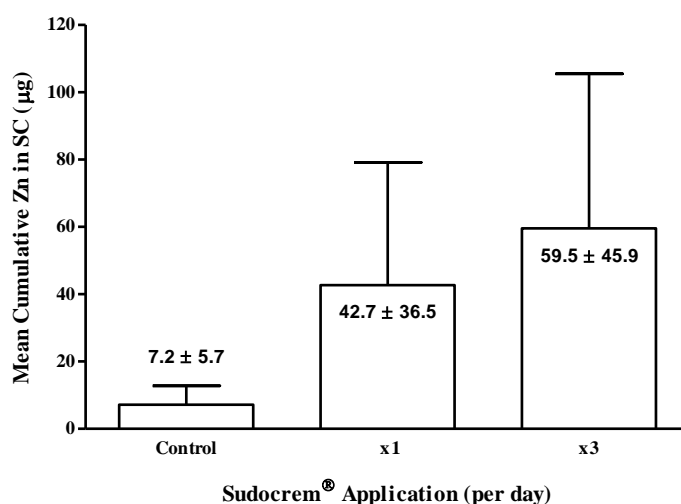


Fig. 7 Cumulative amounts of zinc (mean  $\pm$  SD,  $n = 12$  for the control and  $n = 6$  for x1 and x3 treatments) in stratum corneum treated with Sudocrem® once and three times a day. The control reflects the endogenous zinc level in untreated skin.

## 2. Reverse Iontophoresis

Zinc was extracted at both the anode and cathode chambers by reverse iontophoresis. Overall, as expected, more zinc was extracted from skin treated with Sudocrem® three

times a day than that which received just one. In both cases, the zinc extraction exceeded that from the control, untreated sites (Fig. 8).

#### *Cathode*

Mean cumulative amounts of zinc extracted was  $0.69 \pm 0.37 \mu\text{g}$ ,  $2.79 \pm 1.34 \mu\text{g}$  and  $2.93 \pm 1.74 \mu\text{g}$  from the control, once and three times daily treated sites, respectively. No significant difference, determined by two-tailed paired t-test, was detected between once and three times daily treated skin, where  $P = 0.79$ .

#### *Anode*

Mean cumulative amounts of zinc extracted from the anode chambers of the control, once and three times a day treated skin sites were  $0.27 \pm 0.21 \mu\text{g}$ ,  $0.86 \pm 0.72 \mu\text{g}$  and  $1.12 \pm 0.86 \mu\text{g}$ , respectively. Again, no significant difference was detected by a two tailed-paired t-test on data zinc collected from skin sites treated with Sudocrem<sup>®</sup>,  $P = 0.20$ . Analysis using one-way ANOVA and Tukey's Multiple Comparison test detected a significant difference in levels of zinc between the control and three times a day treated skin only ( $P < 0.05$ ).

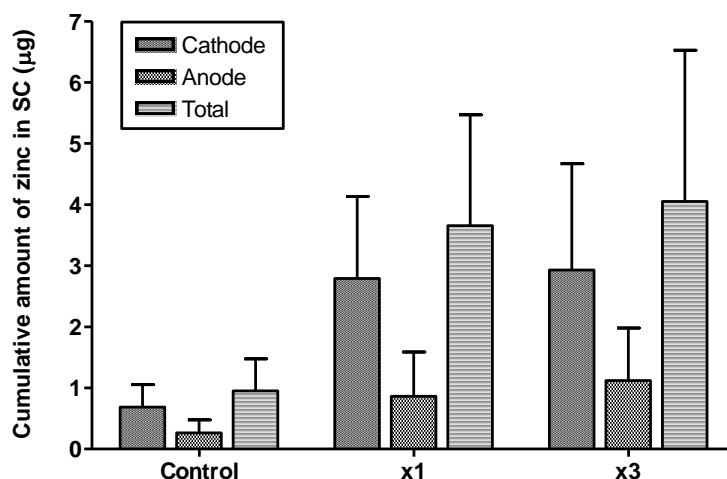


Fig. 8 Reverse iontophoresis extraction of zinc (mean  $\pm$  SD,  $n = 12, 6, 6$  for control, cathodal and anodal extraction, respectively) from untreated skin and from skin treated either once or three times daily with Sudocrem<sup>®</sup>.

### 3. Passive Extraction

Extraction of zinc from the control areas and forearms of volunteers treated with Sudocrem<sup>®</sup> once and three times a day by passive extraction revealed mean cumulative

amounts of  $0.48 \pm 0.33 \mu\text{g}$ ,  $1.23 \pm 0.88 \mu\text{g}$  and  $2.04 \pm 1.38 \mu\text{g}$  of zinc, respectively (Fig. 9). Statistical analyses performed using a two tailed-paired t-test showed a significant difference in levels of zinc detected between once and three times a day treatment ( $P = 0.04$ ).

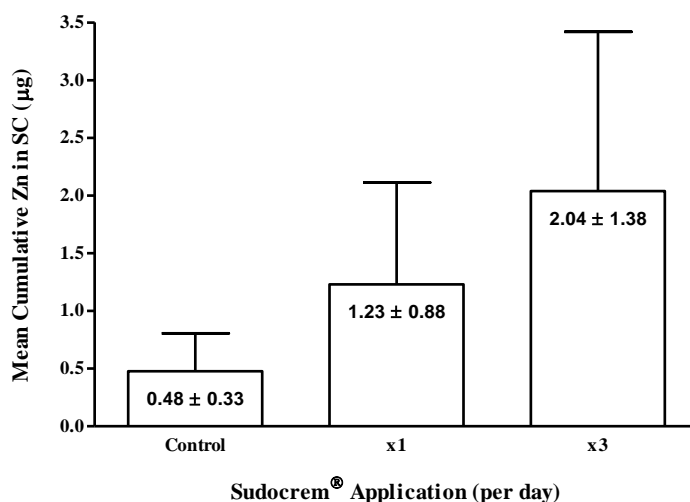


Fig. 9 Mean cumulative zinc (with standard deviations) detected in control, once and three times daily treated skin collected by passive extraction.

### Comparison of Extraction Techniques

By comparing the extraction of zinc from the various skin sites after the different treatments, the efficiency of the methods used can be evaluated.

#### 1. Control

Amounts of zinc extracted by tape stripping, RI cathodal, RI anodal and passive extraction were  $7.15 \pm 5.65 \mu\text{g}$ ,  $0.69 \pm 0.37 \mu\text{g}$ ,  $0.27 \pm 0.21 \mu\text{g}$  and  $0.48 \pm 0.33 \mu\text{g}$ , respectively. One-way ANOVA shows a statistical significance between tape stripping and all other methods of zinc extraction ( $P < 0.0001$ ) (Fig. 10). By separately performing two-tailed paired-t-tests, significant differences between the remaining techniques were established between cathodal and anodal, and anodal and passive extractions with  $P = 0.0004$  and  $P = 0.006$ , respectively. No significant difference was found between cathodal and passive extraction ( $P = 0.11$ ).

## 2. Once a day Sudocrem<sup>®</sup> application

Amounts of zinc extracted by tape stripping, RI cathodal, RI anodal and passive extraction were  $42.7 \pm 36.5 \mu\text{g}$ ,  $2.79 \pm 1.34 \mu\text{g}$ ,  $0.86 \pm 0.72 \mu\text{g}$  and  $1.23 \pm 0.88 \mu\text{g}$ , respectively. A significant difference was detected between tape stripping and other extraction techniques by a one-way ANOVA and Tukey's Multiple comparison test ( $P = 0.001$ ). Two-tailed paired t-tests revealed significant differences between cathodal and anodal, and cathodal and passive extractions, with  $P = 0.01$  and  $P = 0.03$ , respectively. No significant difference was found between anodal and passive extractions ( $P = 0.08$ ). Mean cumulative zinc extraction was highest by tape stripping and least by reverse iontophoretic anodal extraction (Fig. 10).

## 3. Three times a day Sudocrem<sup>®</sup> application

Amounts of zinc extracted by tape stripping, RI cathodal, RI anodal and passive extraction were  $59.6 \pm 45.9$ ,  $2.93 \pm 1.74$ ,  $1.12 \pm 0.86$  and  $2.04 \pm 1.38 \mu\text{g}$ , respectively. Tape stripping was again the most efficient and effective method to extract zinc from the skin (Fig. 10). One-way ANOVA indicated a significant difference between the levels of zinc extracted by tape stripping compared to the other methods used ( $P = 0.0004$ ). Significant differences were also detected between cathodal and anodal, cathodal and passive, and anodal and passive extractions by two-tailed paired t-tests with  $P = 0.01$ ,  $P < 0.01$ , and  $P < 0.05$ , respectively.

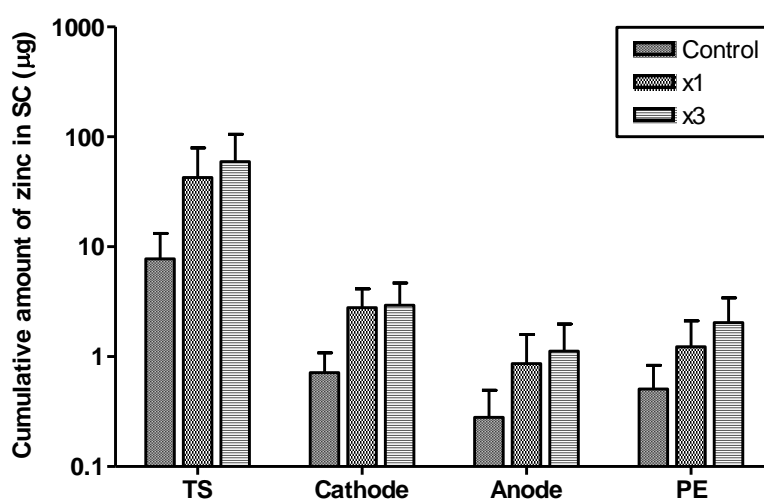


Fig. 10 Mean cumulative amounts of zinc extracted by tape stripping, reverse iontophoresis (cathode and anode) and passive extraction, from a) the control (untreated skin) and skin treated with Sudocrem<sup>®</sup> b) once and, c) three times a day for two weeks.



The amounts of zinc extracted from the skin using the different extraction techniques are collected in Table 1. The highest amounts of zinc were extracted from the skin by tape stripping, the least by reverse iontophoresis at the anode. For the control, once and three times daily applications of Sudocrem<sup>®</sup>, the amounts of zinc recovered by tape stripping ranged from 11-27, 15-50, and 20-53 times greater than the other methods used, respectively.

Table 1 Summary of cumulative zinc (in micrograms; mean  $\pm$  SD) extracted by tape stripping, reverse iontophoresis and passive extraction. P-values shown were derived by two-tailed paired t -tests between once and three times daily application of Sudocrem<sup>®</sup>.

Extraction method	Control (untreated)	x1 Sudocrem <sup>®</sup> application/day	x3 Sudocrem <sup>®</sup> applications/day	P-value (two-tailed paired t-test) (between x1 and x3)	Significantly different?
TS	7.15 $\pm$ 5.65	42.7 $\pm$ 36.5	59.6 $\pm$ 45.9	0.03	Yes
RI cathode	0.69 $\pm$ 0.37	2.79 $\pm$ 1.34	2.93 $\pm$ 1.74	0.79	No
RI anode	0.27 $\pm$ 0.21	0.86 $\pm$ 0.72	1.12 $\pm$ 0.86	0.20	No
RI total	0.95 $\pm$ 0.52	3.66 $\pm$ 1.82	4.05 $\pm$ 2.48	0.50	No
PE	0.48 $\pm$ 0.33	1.23 $\pm$ 0.88	2.04 $\pm$ 1.38	0.04	Yes

## **Discussion**

Prior to this study, the penetration of zinc oxide into human stratum corneum following application of Sudocrem<sup>®</sup> had not been determined.

The results clearly show that tape stripping recovers at least ten-fold more zinc from stratum corneum than either passive or reverse iontophoretic extraction (Fig. 10). Tape stripping recovers both the solubilised zinc in the stratum corneum and also the solid, undissolved form that was sequestered in crevices and furrows of the skin. This material may not necessarily reflect the amount of bioavailable drug since not all the zinc 'recovered' had penetrated into the stratum corneum. This is supported by evidence from previous work where formulations were trapped in skin furrows (19) and appendages. In that study, the dermatopharmacokinetics of betamethasone 17-valerate (BMV) were investigated. Higher levels of the drug were observed when the skin was cleaned with dry paper towel as opposed to an isopropyl alcohol wipe.(19) The experiment revealed that whilst the apparent delivery of BMV appeared to have decreased after using the latter, the diffusivity remained effectively unchanged between the cleaning methods indicating that the high levels of BMV observed from skin dried with a paper towel was due to

sequestered formulation in the furrows.(19) Further, as evident in scanning electron microscopic images of skin treated with various zinc preparations in Chapter 6, formulations were observed on hairs, hair shafts and on the skin surface. Accurate determination of bioavailability is dependent on cleaning procedures. With regards to passive and reverse iontophoretic extractions, these procedures can only recover the zinc dissolved in the skin present in the free ion form. Since zinc oxide is very insoluble - approximately 0.16 mg/100 ml,(20, 21) it follows that the amounts of zinc recovered in the solutions are low. Further, zinc in the solid state will not be extractable either passively or by reverse iontophoresis. As expected, zinc is more efficiently extracted by reverse iontophoresis to the cathode. However, this is dependent on its presence as zinc ions in solution. The differential between the tape stripping results and those by reverse iontophoresis, therefore, between mobile zinc (i.e., that which has become available to the stratum corneum in molecular form) and total zinc trapped in the stratum corneum which includes, mainly, the element on the form of solid zinc oxide.

In summary, tape stripping recovered more zinc from the stratum corneum than passive or reverse iontophoretic extraction. However, not all zinc recovered by tape stripping was that which had penetrated in the stratum corneum. Zinc that was trapped in furrows in the solid state form was also recovered. Reverse iontophoresis at the cathode recovered more zinc than at the anode due to the attraction between positive zinc ions and the negative cathode. Finally, the uptake of zinc was statistically significant between control and three times a day application.

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## **Chapter Eight: Conclusions & Further Work**



## **Chapter Eight**

### **Conclusions**

The main objective of this thesis was to investigate the *in vitro* delivery of zinc from a novel zinc lactate formulation and to compare performance with that of already commercially available zinc oxide preparations. In the process leading up to the percutaneous penetration experiments for zinc, two methodologies were established to firstly enable the assessment and characterisation of skin barrier function, and secondly, analysis of zinc in the stratum corneum to be quantified by extraction of the tape strips. The former was achieved using minimally invasive procedures; tape stripping and transepidermal water loss measurements, and the latter, by tape stripping and analysis of samples by atomic absorption spectroscopy or inductively coupled plasma-atomic emission spectroscopy. The methodologies developed were effective, simple, non-invasive and inexpensive. Further, the findings of Chapter 2 - the short term application of Aqueous Cream BP caused skin atrophy and increased percutaneous penetration, was published in the British Journal of Dermatology and is already impacting the way Aqueous Cream BP is used for atopic eczema as an emollient.(1)

The results obtained in Chapter 4 confirmed what numerous previous studies had reported; that the percutaneous penetration of zinc into the skin was, at best, minimal and was mainly detected in the outer layers of the stratum corneum. The investigation of commercial preparations in this thesis provide further evidence that formulation of a novel treatment for eczema that is able to deliver zinc to the skin more efficiently is needed.

Development of the novel 1% w/w zinc lactate formulation in Chapter 5 afforded increased penetration of zinc into the skin. The results show that zinc uptake, when represented as percentage of the applied dose, from 1% w/w zinc lactate cream, was higher than all formulations tested (i.e., aqueous zinc chloride solutions, zinc and castor oil, Sudocrem<sup>®</sup>, Morhulin<sup>®</sup> and 2% w/v zinc lactate). The rationale behind this could be due to (i) the use of penetration enhancers or detergents that would aid the absorption of zinc into the skin, (ii) the use of small particle size, since these particles would penetrate more easily into the skin, and (iii) the incorporation of oil into the formulation would occlude the surface of the skin which would also enhance percutaneous absorption.(2)



In Chapter 6, the distribution of zinc on the surface of the skin and the effects of different cleaning procedures, (wiping with a dry paper towel versus rinsing under deionised water), on zinc disposition were studied. Scanning electron microscopy (SEM) was used to image the skin before and after treatment with various zinc preparations. Passive diffusion with 5% and 50% w/v  $\text{ZnCl}_2$  showed that the higher concentration solution left visible deposits on the surface of the skin and on hairs. These deposits were not present after the rinsing the skin under water indicating that the substance may have well been  $\text{ZnCl}_2$  since it is highly water soluble. Further, these deposits were not observed on the control (untreated) skin. The images also showed that the application of Morhulin<sup>®</sup> ointment left a thin coating on the surface of the skin which, even after being cleaned with an isopropyl alcohol swab, was visible across the entire skin surface. Energy dispersive x-ray (EDX) spectroscopy and element mapping was used to qualitatively examine the amounts and distribution of zinc on the skin. Both EDX spectra and element maps showed that the concentration of zinc applied to the skin correlated to the amounts present on the surface. Both techniques were also used to compare amounts of zinc on the skin surface after cleaning with dry paper towels and rinsing under deionised water. Spectra and element maps showed that washing with deionised water removed relatively large quantities of zinc from the skin. Finally, element mapping showed that zinc did not have any affinity for a particular site on the skin and that it was distributed evenly across the surface.

The final experimental chapter involved the *in vivo* testing of Sudocrem<sup>®</sup> (15.25% w/v  $\text{ZnO}$ ) on human forearms and the elucidation of the most effective method for zinc extraction from the skin using tape stripping, reverse iontophoresis and passive extraction. Tape stripping proved to be the most efficient method for the extraction of zinc from the skin. The higher levels of zinc measured using this method was most likely a result of extracting zinc from formulation that was sequestered in skin furrows and other appendages together with zinc that was solubilised in the skin. With regard to reverse iontophoresis and passive extraction, the poor extraction efficiency could be explained by the fact that only zinc in the free ion form could be extracted from the skin and none from the semi-solid preparation remaining on the skin in crevices and appendages.

In conclusion, methods for assessing and characterising skin barrier function, extraction and quantification of zinc in skin have been successfully developed. The *in vivo* application of Sudocrem<sup>®</sup> to human stratum corneum has shown that limited quantities of zinc were delivered into the skin. The study also indicated that tape stripping extracted the

highest amounts of zinc from the skin followed by cathodal reverse iontophoresis. Scanning electron microscopy, EDX spectroscopy and element mapping allowed the distribution and relative quantities of zinc in the skin to be examined. The effects of cleaning procedures on the concentration of zinc on the skin and effects on skin structure were also deduced.

Finally, preliminary research has shown that zinc can be delivered (albeit quite poorly) to the skin and that the percentage of the applied dose of zinc delivered from commercial preparations *in vitro*, zinc chloride and 2% w/v zinc lactate ranges from 0.004 - 0.04%. The formulation of a 1% w/w zinc lactate cream increased delivery to the skin by approximately double that of previously tested formulations (0.07% of the applied dose). The novel topical formulation for the treatment of the skin barrier in atopic eczema is therefore a promising starting point since not only can more zinc be delivered from the formulation to inhibit protease activity, suppress colonisation of *S.aureus* and aid wound healing but it also benefits the skin by restoring the natural pH which increases the production of sphingosine, providing hydration to the stratum corneum and decreasing scaling.(3-8) It is clear that the formulation offers many beneficial properties that can treat symptoms of eczema and aid barrier repair, however, further investigation and development of the formulation is needed, before a commercially useful formulation can be considered.

### **Further Work**

Detrimental effects of zinc deficiency on the skin barrier have been well documented and research has shown that zinc can aid in wound healing. In the development of a novel topical formulation, it is important that the therapeutic dose is known. Therefore, it is proposed that further investigations are needed in this respect. This could be achieved by the application of various concentrations of zinc on controlled wounds and recording the rate of healing. Optimum zinc concentration for the formulation could be deduced in this manner.

Since the experiments carried out in this thesis have been conducted on the intact skin barrier (*in vitro* and *in vivo*), the percutaneous penetration of zinc through damaged skin should be investigated. The simplest and least expensive means by which this could be achieved is to perturb the stratum corneum via tape stripping.(9) This would, in effect, allow the stratum corneum to be partially bypassed and therefore, the compromised skin barrier that occurs in eczema to be mimicked and a more accurate representation of zinc uptake to be deduced.

As mentioned previously, sodium lauryl sulphate (SLS) is a chemical known to alter the skin barrier and has been used in many investigations to study the penetration of substances through damaged skin.(10-12) For *in vivo* studies, as discussed in Chapter 2, the application of SLS at approximately 1% concentration should suffice to induce changes in the skin barrier and increase permeability to mimic the damaged stratum corneum in eczema.

Further, the use of iontophoresis to deliver zinc to the skin could be explored.(9, 13, 14) This method may be utilised directly on eczematous lesions to deliver zinc to the skin to aid healing of the wound and restoration of the skin barrier. This method would require the zinc to be in free ion form and a suitable vehicle would be necessary. It is a relatively pain free means to deliver therapeutic levels of zinc directly and fairly rapidly to affected sites.

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